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OBITUARY NOTICES.

THORBURN BRAILSFORD ROBERTSON (1884–1930).

THORBURN BRAILSFORD ROBERTSON, Professor of Biochemistry and General Physiology at the University of Adelaide, and Director of the Animal Nutrition Laboratory of the Commonwealth Council for Scientific and Industrial Research, died from pneumonia at Glenelg, South Australia, on the 18th January, 1930.

Biological science has lost, in Robertson, one of its most brilliant and energetic exponents. It is impossible, in a short note, to discuss the many problems which at different times absorbed his interest. He was one of those vigorous, fearless thinkers who seem to be entirely free from the inhibitions which restrain most of us in narrow paths of scientific enquiry.

His magnificent courage and imagination led him into every aspect of biology which presented problems to his consciousness. Perhaps he attempted more than he could possibly expect to accomplish. His industry was prodigious and his output was remarkable, for he suffered ill health throughout his life and died at the comparatively early age of 46.

His work in recent years has been mainly concerned with the general problem of growth, but he had unusually broad interests and his publications covered a great variety of subjects. The first investigation on which he worked intensively was the physical chemistry of the proteins. He was a pioneer in this field and was one of the first workers to show that protein chemistry was amenable to quantitative treatment. The value of his early experimental work on the binding power of proteins for bases has been recognised on all sides, though his theoretical conceptions on the nature of protein salts failed to gain adherents. The problem of growth in all its aspects was his next chief interest, but he never suffered mental confinement in any one compartment of science. He went without hesitation into all phases of general physiology. At the time of his death he was working hard at animal nutrition, as Director of the Waite Research Institute at Adelaide, and his contributions to the biochemical aspects of sheep farming in Australia promise to be of considerable economic significance for the Dominion.

He was a fluent, lucid and inspiring writer. His textbook of Biochemistry is unique in the success with which the subject matter is conveyed to the reader through the personality of its author. This book was written in two or three months with practically no reference to notes or books, and differs as widely as possible from the usual compilations which serve the purpose of textbooks. It is not only one of the most readable, but also one of the most stimulating works on the subject.

Among his voluminous scientific writings are many which deal with the social relations of science, and no pen has more eloquently expressed the claims of the history of science to a place among the humanities. He once

wrote a fascinating description of human biochemistry for little children under the intriguing title of the *Universe and the Mayonnaise.*

Robertson had a most attractive personality, and was beloved by his colleagues in California, Toronto and Adelaide. Like all strong characters he made enemies, but never among his close associates. His originality of mind made him a stimulating colleague, and to his students he was as gracious as he was inspiring.

His enthusiasm was communicated to those around him, and his return to Adelaide in 1919 to succeed Sir Edward Stirling in the Chair of Physiology was followed by an expansion of interest in scientific research, not only in Adelaide but throughout the Dominion. Robertson founded the *Australian Journal of Experimental Biology and Medical Science,* to which he was a frequent contributor.

Robertson's main service to science was in the realm of ideas. His practical work was of lesser significance, but in almost every direction in which his imagination led him he either contributed new conceptions or reoriented prevailing ideas.

Biochemistry at this period has an abundance of practical workers, but a dearth of vision. Men of Robertson's rare quality can ill be spared.

H. W.

ROBERT LAUDER MACKENZIE WALLIS (1886–1929).

Mackenzie Wallis was a Cambridge man, a scholar of Downing College, with a first class in the Natural Science Tripos, Part I. His first appointment was that of Lecturer in Chemical Physiology at Cardiff and his second that of Demonstrator in Chemical Pathology at St Bartholomew's Hospital. Soon after the war began Mackenzie Wallis went to India with the rank of Captain, where he remained to the end of the war. On his return in 1919 he was appointed Lecturer in Chemical Pathology at St Bartholomew's in succession to Sir A. E. Garrod, and in the same year he was awarded the M.D. degree at Cambridge for a thesis which gained him the Horton-Smith Prize. In 1921 he was appointed Chemical Pathologist to the Hospital.

Mackenzie Wallis was a hard worker; he organised the new and rapidly growing department of Chemical Pathology at St Bartholomew's and carried out a great mass of routine work in this subject himself, and supervised the work of others. The experience he gained in this way was placed at the service of his colleagues and of many others who were glad to avail themselves of it. He was constantly engaged on research in Chemical Pathology and the results of this work will be found in various clinical journals. Mackenzie Wallis was a genial and charming man, always ready to impart his knowledge to others and as ready to learn from others: his death on April 1st, 1929, at the age of 43 years, was a severe blow to his many friends at St Bartholomew's Hospital and elsewhere.

W. H. H.

LXIV. THE REDUCTION OF METHYLENE BLUE IN MILK.

THE INFLUENCE OF LIGHT.

By HUGH ROBINSON WHITEHEAD.

From the Dairy Research Institute, Massey Agricultural College, University of New Zealand.

(*Received March 27th, 1930.*)

THREE types of reaction which will bring about the reduction of methylene blue added to milk have been known for a considerable time and have been utilised in various ways.

(i) Schardinger [1902] has shown that milk contains an enzyme which is able to catalyse the reduction of methylene blue in the presence of an aldehyde. Trommsdorff [1909] has proved that the enzyme is a constituent of the milk itself, and is independent of micro-organisms. Other observers have shown that aldehydes may be replaced by xanthine, and methylene blue by nitrates. Hence the reaction is now recognised as a typical oxidation-reduction in which hydrogen donator and hydrogen acceptor are enabled to interact through the presence of an enzyme or catalyst.

(ii) Milk which has been heated for a short time to a temperature over 100° will bring about the decoloration of methylene blue. The mechanism of this reaction has not yet been definitely established. The possibility of enzyme action is of course excluded. Barthel [1925] considers that an oxidation-reduction occurs in which citrate acts as hydrogen donator, methylene blue as hydrogen acceptor, and some inorganic constituent of milk as catalyst. Schwartz [1929], however, has brought forward evidence in favour of the theory that hydrolytic products of the milk proteins, probably those containing sulphydryl groups, are the reducing agents.

(iii) Milk containing living bacteria will decolorise methylene blue. The reaction in this instance is caused by an enzyme associated with the micro-organisms, and most probably is an oxidation-reduction in which the methylene blue acts as a hydrogen acceptor. The corresponding oxidation, which occurs simultaneously, must be a reaction which supplies energy for the growth of the organisms. Clark, Cohen, and Gibbs [1928] have shown that a reaction takes place in the absence of methylene blue, and that the dye, if present only in small quantity, is merely an indicator of reducing potential, analogous to an indicator of hydrogen ion concentration. Milk has therefore no specific function in the reaction, but is merely the source of substances suitable for the supply of energy to bacteria.

The third reaction has been used for many years as a means of determining the degree of contamination of market milk supplies, and it was while applying it for this purpose that the observation was made that light has a significant effect on the progress of the decoloration of methylene blue in milk. Some samples of milk to which methylene blue had been added were incubated in a water-bath with glass sides, designed to facilitate observation of the tubes. All the samples gave unexpectedly short reduction times averaging 2 to 3 hours. On repeating the test with the same samples of milk, but using an ordinary metal water-bath, many of the samples took more than 7 hours to reduce. Sunlight evidently had influenced the progress of the decoloration.

So far as can be ascertained, there is no reference in the literature on methylene blue reduction in milk to any observation of the effect of light. Clark, Cohen and Gibbs [1928] have observed an oxidising effect of ultra-violet light on methylene white in aqueous solution, and Webster, Hill and Eidinow [1924] have used the decoloration of methylene blue in the presence of acetone for measuring the intensity of ultra-violet light. The latter reaction is said to be due to the decomposition of the acetone with the formation of reducing substances.

Experiments were devised in an attempt to find an explanation of the mechanism of the process in milk. It was necessary first to determine whether an enzyme of the milk or of the contaminating organisms was concerned in the reaction. Reductase tests were set up in duplicate with fresh milk and some of the same milk heated for half an hour in a boiling water-bath. One series was incubated in water at 37° in a thin-walled glass vessel exposed to bright sunlight, and the other series was incubated in darkness. The tubes which were not exposed to sunlight retained the blue colour for more than 7 hours. In the series exposed to sunlight there was very little difference between the heated and unheated milk samples. They showed decoloration in times which varied from half an hour to several hours, according to the intensity of the sunlight. Usually the heated milk samples were decolorised in rather shorter time than the corresponding unheated samples, but this was not always the case, and probably the difference was due to the loss of dissolved oxygen brought about by heating, and the varying degree of subsequent aeration. In every instance the lower part of the tube was the first to lose the blue colour and the decoloration progressed upwards. There was always a blue ring left at the top of the tube and on dull days the decoloration was never complete in the upper half of the milk. In particularly intense sunlight the decoloration proceeded until there was merely a blue ring at the milk surface. Thereafter the blue colour was slowly regenerated in the upper part of the tube, and sometimes reached halfway down the milk column after several hours. In some experiments a layer of liquid paraffin was superimposed on the milk to avoid any influence of atmospheric oxygen, but with heated milk no difference was observed. In unheated milk decoloration

occurred a little more rapidly when the milk was covered with paraffin, but the differences were hardly significant. This is in accord with the findings of Thornton and Hastings [1929] that diffusion of atmospheric oxygen into milk is much too slow to affect the reductase test. The number of bacteria in the milk seemed to have no bearing on the phenomenon in unheated milk, so long as the reduction caused by the organisms was sufficiently delayed, so that it was not superimposed on the light effect. In some experiments milk was obtained with sterile precautions directly from the cow into a sterile flask and the test was performed immediately. The results were exactly the same as those obtained with milk several hours old. This rules out the possibility that some heat-stable substance produced by bacterial action is necessary for the reaction.

It is evident therefore that the reaction takes place between some chemical constituent of the milk and methylene blue under the influence of sunlight, or that some reaction takes place in the milk itself which produces a reducing potential sufficient to reduce methylene blue. In the latter case the dye would be acting merely as an indicator of the oxidation-reduction potential; in the former case it would be essential to the progress of the reaction. Electrometric measurements of the oxidation-reduction potential in the presence and the absence of the dye will be necessary to decide which explanation is correct.

In a consideration of the substances in milk which may be concerned in the reaction, the milk-fat attracted attention, for it is well known that some of the unsaturated fats in cream undergo oxidation more readily in the presence of light. To determine whether the fat was responsible for the reducing action, milk was run through a centrifugal separator and reductase tests were performed on the original milk and on the separated milk. In bright sunlight the tubes containing separated milk suffered no decoloration, whereas the whole milk tubes became colourless as usual in a longer or shorter time, depending on the intensity of the light. The blue sometimes paled temporarily in separated milk, but returned in an hour or two to its full depth. A layer of liquid paraffin on the milk again had no effect, so that the greater ease of diffusion of oxygen into separated milk could not be the explanation of the absence of decoloration. The substance necessary for the reaction must be a constituent of the cream.

As a further test of the fat oxidation hypothesis some experiments were carried out in which salts of known fatty acids were added to separated milk in an endeavour to replace the substance which reacts with methylene blue. The sodium salts of palmitic and oleic acids were chosen as representatives of the saturated and unsaturated types. Clear-cut differences were immediately observed in reductase tests incorporating these substances and separated milk. The sodium palmitate had no effect within 7 hours, while the sodium oleate induced reduction in a short time in sunlight and had no effect in darkness. This seems strong evidence in favour of the theory that the

reduction of methylene blue in milk under the influence of sunlight is an oxidation-reduction phenomenon in which unsaturated fats are oxidised and methylene blue acts as hydrogen acceptor. The occasional return of colour in strong sunlight in the top layers of milk where fat is collecting may possibly be due to an autoxidation of the oxidised fats resulting in a final oxidation-reduction potential sufficient partly to re-oxidise the methylene blue. Further evidence would be needed on this point.

Further work is projected in which the phenomenon will be examined in a more quantitative manner with the aid of a quartz mercury-vapour lamp, for it seems most probable that the active agent is ultra-violet light and that more powerful effects will be observed than with sunlight acting through one or two layers of glass. The phenomenon has obvious practical importance in connection with the keeping qualities of butter-fat in the presence of hydrogen acceptors.

Experimental.

In all reductase tests 20 cc. of milk were measured into a 6″ by $\frac{5}{8}$″ test-tube and 1 cc. of methylene blue solution was added. The dye solution was prepared from tablets supplied by Blauenfeldt and Tvede, Copenhagen, for the purpose of determining the bacterial reduction of methylene blue in market milk. The final concentration of dye in the milk was about 1 in 100,000. The tubes used had been previously sterilised and when the tubes were inverted to mix the contents, the operator's finger was washed with alcohol on each occasion. The samples were exposed to sunlight by immersing them in water at 37° in a thin beaker set close to the laboratory window.

The effect of sunlight on methylene blue reduction in heated and unheated whole milk.

Milk was measured into test-tubes some of which were immersed in a boiling water-bath for half an hour. The heated samples were thereafter cooled in water and inverted several times to aerate the milk to a moderate degree. Methylene blue was added to all the tubes and one set was incubated exposed to sunlight while a control set was incubated in darkness.

Table I. *Times of decoloration in hours.*

	In the light		In the dark	
	Unheated milk	Heated milk	Unheated milk	Heated milk
Bright day	2·25	1·75	All over 7	All over 7
Bright day	1·5	0·5	,,	,,
Dull day	1·5 (only $\frac{1}{2}$ decol.)	1·5 (only $\frac{1}{2}$ decol.)	,,	,,
Dull day	4 (only $\frac{1}{2}$ decol.)	4 (only $\frac{1}{2}$ decol.)	,,	,,
Bright day	1·5	1·75	,,	,,

The above results are typical of many observations. On dull days the decoloration usually progressed no farther when the lower half of the milk

column was colourless. The variation between unheated and heated milks is probably accounted for by different degrees of aeration. The time of decoloration may also vary with different milks.

The effect of sunlight on methylene blue reduction in whole and separated milk.

Milk was run through a sterilised centrifugal separator and both the original milk and the separated milk were used for reductase tests. Both milks were used in the heated and unheated states and in some tubes a layer of liquid paraffin was added. Bacteriological tests on the milk before and after separation showed that no measurable contamination had taken place.

Table II. *Times of decoloration in hours, in the light.*

	Whole milk	Whole milk +paraffin	Whole milk heated	Whole milk heated +paraffin	Separated milk	Separated milk +paraffin	Separated milk heated	Separated milk heated +paraffin
Bright day	2	0·5	0·5	0·5	>7	>7	>7	>7
Dull day	1·5 (only ¼ decol.)	2 (only ½ decol.)	1·5 (only ½ decol.)	3·5 (only ½ decol.)	>7	>7	>7	>7
Dull day	4 (only ½ decol.)	4 (only ¾ decol.)	4 (only ½ decol.)	4 (only ½ decol.)	>7	>7	>7	>7
Bright day	1	—	—	—	>7	—	—	—
Bright day	1·5	—	—	—	>7	—	—	—

All the controls incubated in darkness were not decolorised in 7 hours. The difference between whole milk and separated milk was always clear cut and it is evident that diffusion of atmospheric oxygen has no appreciable effect.

The effect of the addition of salts of fatty acids to separated milk.

Milk was freed from fat as previously described. Sodium oleate and sodium palmitate were added in amounts of approximately 0·5 g. to different tubes of whole and separated milk, and partially dissolved by gentle shaking. Reductase tests were set up as before.

Table III. *Times of decoloration in hours, in the light.*

	Whole milk	Separated milk	Whole milk +Na oleate	Whole milk +Na palmitate	Separated milk +Na oleate	Separated milk +Na palmitate
Bright day	1·75	>7	1	1·25	0·5	>7
Bright day	—	>7	—	—	1·5	>7

All the controls incubated in darkness were not decolorised in 7 hours. These are typical of several experiments on similar lines. There is thus strong evidence that an unsaturated acid such as oleic acid can replace some constituent of the cream in this reaction with methylene blue.

SUMMARY.

1. Methylene blue added to fresh milk of good quality is reduced in a short time in the presence of sunlight at 37°. In darkness at 37° no decoloration occurs within 7 hours.

2. The reaction in sunlight is not due to an enzyme, for it proceeds equally well in milk which has been heated to 100° for 30 minutes.

3. Milk from which the fat has been removed by centrifugal separation no longer gives the reaction, but the activity of the milk can be restored by an addition of sodium oleate. Sodium palmitate has not a similar action.

4. It is suggested that sunlight catalyses an oxidation-reduction reaction in which unsaturated fats are oxidised and methylene blue is reduced.

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LXV. THE TRACES OF COMBUSTIBLE GASES IN HUMAN EXPIRED AIR.

By THOMAS RICHARD PARSONS.

From the Laboratory of Zoophysiology, University of Copenhagen.

(*Received April 9th, 1930.*)

The stimulus to the present investigation was furnished by the observations of Campbell [1929], who showed that the gases present in the peritoneal cavities of animals may contain as much hydrogen (produced by intestinal putrefaction) as corresponds to a tension of the order of 20 mm. Hg. Some of this hydrogen must dissolve in the blood and later diffuse through the lungs into the expired air. The object of the experiments herein recorded has been to determine the extent to which such a transfer of hydrogen or other combustible gas takes place in the human subject, in order to decide whether this constitutes a factor necessary to be taken into consideration in the calculation of the total respiratory exchange from observations carried out with an open circuit respiration chamber. In using such an apparatus the volume of the ingoing air is calculated from the percentage of nitrogen in the outgoing air, the total amount of nitrogen being assumed to be unaltered during its passage through the chamber. But any hydrogen given off from the subject would be returned as unabsorbable gas, *i.e.* nitrogen, in the analysis of the outgoing air and would influence the calculation of the volume of air entering the chamber and so of the respiratory exchange of the subject.

The possibility of carrying out such an investigation depended on the circumstance that there was available the gas analysis apparatus accurate to 0·001 % already described by Krogh [1920]. In using such an apparatus for the estimation of minute traces of combustible gases, two points of technique must be carefully observed. Firstly, the solubility of oxygen and nitrogen in the potash solution used for the CO_2 absorption becomes a factor of very significant magnitude: in order to avoid errors from this source, it is usual to keep this potash solution saturated with air at the temperature of the apparatus, but this restricts the use of the apparatus to the analysis of gas mixtures not differing very appreciably in composition from atmospheric air. Then, secondly, it has already been shown [Krogh, 1919] that pure atmospheric air itself shows a just measurable diminution in volume amounting to about 0·0005 cc. for each minute of exposure to the hot platinum wire of the combustion pipette—an effect that cannot be due to the presence of combustible gases in the atmosphere as it can be repeatedly obtained with the same sample of air. This means that there will never be an absolutely final end-point to a

combustion analysis; when all the combustible gases present have been burned, a further slight diminution of volume will always be obtainable on continuing the combustion. The diminution of volume due to this effect in pure air becomes added to that due to the burning of any combustible gases that may be present. These considerations led to the adoption of the following technique.

In order to have the maximum amount of hydrogen for measurement we analysed not the expired air as directly collected but air that had been rebreathed until its hydrogen tension had come into equilibrium with that in the blood. A simple closed circuit was used consisting of a pair of respiratory valves, a tower of loosely packed soda-lime and a Douglas bag. Into the latter there were drawn through a cotton-wool filter about 5 l. of outside air and to this was added enough oxygen from a cylinder to cover the estimated needs of the subject during the experiment. In this way the composition of the air in the bag at the end of the rebreathing period did not differ greatly from that of the atmosphere, and solubility errors did not enter into the analysis. It was found convenient to observe the volume of air in the bag by attaching a sheet of metal to its upper surface and connecting this by a thread to a simple lever: in this way it was easy to see the point at which all the added oxygen had been used up by the subject, which was the point at which the rebreathing was stopped. To ensure that the results were not being affected by possible contaminating traces of combustible gases, both the air and the oxygen used were passed slowly through about 6 cm. of red-hot wire-form CuO before being collected in the bag. As a further precaution in each experiment a control sample of air was withdrawn from the bag for analysis before the oxygen was added. It was also proved that the rubber lining of the bag did not give off any appreciable amount of combustible gas to air stored within it. Lastly, in order to avoid any possible formation of hydrogen by the action of moisture on oxidisable metals, no metal parts other than the heavily nickel-plated valves were used in the respiration circuit. Every effort was made to carry out the analyses as uniformly as possible. In all cases after a preliminary absorption of any CO_2 present the gas was passed to and fro for 6 minutes (in three carefully measured periods of 2 minutes) over the platinum wire of the combustion pipette heated to dull redness. After measurement of the resulting contraction a second CO_2 absorption was carried out.

Table I contains the results of duplicate determinations made in this way on control samples of atmospheric air collected and treated as mentioned in each case. It will be seen that these data confirm the former statement that in pure air for each minute of exposure to the hot wire shrinkage occurs to the extent of about 0·001 % of the original volume, but that the volume of the potash-absorbable product apparently formed is always less than twice the contraction on combustion. This indicates that, whatever its explanation, the phenomenon cannot be due to the presence of traces of carbon monoxide in the air analysed and this is confirmed by the observation that the effect

is equally well obtained with air that has been passed over red-hot copper oxide.

Table I. *Control combustions with atmospheric air.*

Date	Material analysed	× 0·01 % of volume taken for analysis (50 cc.) Contraction on 6 mins. combustion	Subsequently absorbed by KOH
3. v	Filtered outside air	0·50	0·85
		0·35	0·80
7. v	Air after exposure to red-hot CuO	0·50	0·50
		0·50	0·65
10. v	Filtered outside air	0·40	1·10
		0·50	0·90
10. v	Air after storage in Douglas bag 6·5 hours	0·70	0·75
		0·65	0·75
13. v	Air after storage in Douglas bag 3 days	0·80	1·00
		0·65	1·00
15. v	Filtered laboratory air	0·55	0·65
		0·25	0·55
19. v	Filtered outside air	0·50	0·55
1. vi	Filtered outside air	0·45	0·75
4. vi	Air from compressed air supply after exposure to red-hot CuO	0·50	0·85

Table II. *Analyses of rebreathed air.*

Date	Experiment and subject	× 0·01 % of volume taken for analysis (50 cc.) Contraction on 6 mins. combustion	Subsequently absorbed by KOH	H_2	CH_4
30. iv	Filtered air + 5 l. O_2. Rebreathed 15 mins. Subject P.	1·00	1·20	0·25	0·10
		1·00	0·65		
2. v	Filtered air + 5 l. O_2. Rebreathed 15 mins. Subject N.	1·20	1·35	(?)	0·55
		1·50	1·40		
15. v	Air + 5 l. O_2 passed over red-hot CuO. Rebreathed 15 mins. Subject R.	4·75	0·90	2·80	0·00
		4·55	0·70		
18. v	Air + 5 l. O_2 passed over red-hot CuO. Rebreathed 15 mins. Subject P.	1·90	1·20	0·25	0·55
		2·00	1·00		
31. v	Air + 6 l. O_2 passed over red-hot CuO. Rebreathed 15 mins. Subject R.	4·00	1·20	1·85	0·35
		3·90	1·00		
4. vi	7·5 l. air + 20 l. O_2 all passed over red-hot CuO. Rebreathed 63 mins. Subject P.	2·15	1·45	0·30	0·60
		1·15 (?)	1·35		

Turning now to the analyses of the rebreathed air recorded in Table II, it will be seen at once that the contractions on combustion and the subsequent amounts of absorption by potash are invariably larger than with atmospheric air, indicating that traces of combustible gases are in fact expired by the human subject. The subjects used for these observations were all examined in the absorptive period either in the middle of the morning (Exps. 1, 2 and 4) or after lunch (remaining experiments). That these traces of expired combustible gases do not consist of carbon monoxide derived from tobacco smoke is evident from the fact that the highest figures were obtained with the subject R. who is a non-smoker. Prolongation of the rebreathing period from

15 minutes to 63 minutes as in the last experiment recorded led to no significant increase in the amount of combustible gas collected.

In the last two columns of Table II the amounts of combustible gases in the rebreathed air have been calculated on the assumption that they consist of hydrogen and methane only. In making this calculation the contraction on combustion and subsequent absorption by potash obtained on the same day with a control sample of atmospheric air, collected as mentioned from the Douglas bag just before each experiment, were subtracted from the corresponding data obtained with the rebreathed air.

In order to determine approximately the rate at which these gases are given off from the body, let us take the highest percentage of combustible gas obtained, namely the $2{\cdot}80 \times 0{\cdot}01$ % from the experiment of 15. v. This appears to be made up entirely of hydrogen. This percentage corresponds to a tension in moist air at body temperature of approximately 0·2 mm. Hg. Taking this as the same as the tension of hydrogen in the mixed venous blood, and assuming an average rate of blood flow through the lungs of 5 l. per minute and a solubility of hydrogen in blood equal to that in water under the same conditions, the amount of hydrogen carried to the lungs per minute works out to be 0·02 cc. But during a respiratory experiment on a human subject about 60 l. of air pass through the chamber per minute, so that if the blood gave off the whole of its hydrogen while passing through the lungs this would add only 1 part of inert gas to 3×10^6 parts of outgoing air—a change in composition that is about one-thirtieth of the smallest detectable by the most accurate technique yet devised. We may conclude with certainty, therefore, that, whatever may be the exact nature of these traces of combustible gases in human expired air, even if they were produced at 10 times the maximum rate here observed, they would be quite without influence on the results of the most accurate respiratory experiments that have so far been made.

Summary.

1. The slight shrinkage in volume when pure atmospheric air is submitted to combustion analysis is confirmed. This effect cannot be due to the presence of traces of ordinary combustible gases as it is obtained in air that has been passed over red-hot copper oxide.

2. Air that has been rebreathed by a human subject contains traces of combustible gases in amounts that indicate a rate of production of about 0·02 cc. per minute. This is far too small to be of significance in determinations of total respiratory exchange.

It is a great pleasure to the author to acknowledge here the stimulating help of Professor Krogh during the carrying out, at his suggestion, of this investigation.

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LXVI. THE INFLUENCE OF ARGININE UPON THE GROWTH RATE OF A TRANSPLANTABLE TUMOUR IN THE MOUSE.

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Research in nutrition has shown that the various amino-acids differ among themselves in respect of their relative importance in relation to growth in the immature animal, and to maintenance of weight in the adult [Mitchell and Hamilton, 1929].

The results of Ackroyd and Hopkins [1916] provided evidence that arginine and histidine are interconvertible in the body, and that the presence of one or the other is essential for the processes of growth; that arginine is necessary for the maintenance of weight had been demonstrated by Geiling [1917] in the mouse. Confirmation of the indispensability of histidine was obtained by Rose and Cox [1924], but they reported that arginine, as a dietary constituent, was without effect on weight; similar results were recorded by Rose and Cook [1925]. The results obtained by Stewart [1925] were in agreement with the latter in respect of histidine, but with regard to the significance of arginine they were intermediate between the results of Ackroyd and Hopkins, and those of Rose and Cox. From these more recent investigations it appeared that arginine and histidine were not mutually replaceable in metabolism, and that arginine was definitely inferior to histidine in its ability to maintain weight, or as a promoter of growth. Owing to variations in the amount of food assimilated the interpretation of feeding experiments must often be a matter of difficulty, and the criterion of the capacity of a substance to induce growth is its effect upon the animal as a whole. It may be assumed that the amino-acid requirements of the various organs and tissues of the body differ in accordance with differences in specific function, and with the chemical composition of their structural units, the cells of which they are composed. It has been suggested that histidine is necessary for nuclear synthesis [Rose and Cook, 1925], and the researches of Kossel [1928] indicate that arginine may be an important constituent of nuclear substance as in the case of sperm. Edlbacher and Merz [1927], have shown that the arginase content of tumours exceeds that of most normal tissues, and it may be noted that the arginine content of neoplastic tissue, as estimated by Sakaguchi's reaction [1915] is also relatively high. Therefore it appears that arginine is present in considerable amount in tumours, and

that such tissues are well equipped for its catabolism, but unless they are capable of synthesising this amino-acid they must obtain it from their host; if arginine were present in excess of the nutritive requirements of the latter it would be available for the purpose of nuclear synthesis and cell proliferation, and this should be manifested by an increase in the rate of tumour growth.

It was the object of this investigation to determine whether arginine had any influence upon tumour growth-rate, and whether it was distinct from other amino-acids in exhibiting this effect.

Material.

The mice used for the experiments were ordinary, healthy, unselected stock, representing a mixed population; they were 2–3 months old at the time of implantation, and an approximately equal number of males and females were included in each experimental group. They were all fed alike on a diet of brown bread, flaked maize, and lettuce (when available). It may be of interest to note in connection with diet that the arginine content of brown bread, as estimated by Sakaguchi's reaction, is quite considerable, and both treated and control groups were given as much as they would eat.

The tumour was a transplantable mouse carcinoma, for which I am indebted to Mr J. J. M. Shaw, of the Department of Surgery, Edinburgh University. This tumour (M 63), is palpable about ten days after implantation, often earlier, and attains a large size in a period of three weeks. It produces no metastases, and therefore the mice remain perfectly well.

Method.

Implantation was performed by means of an implantation needle, strict aseptic precautions being observed.

The amino-acids[1] were administered by hypodermic injection at some distance from the site of implantation.

Exp. I. Implantation was carried out as described, and after ten days all mice bearing palpable tumours on that date were segregated, divided into groups, and treatment was commenced. This consisted in the injection of 0·3 cc. of a 10 % solution of the amino-acid allotted to the group, and treatment was repeated every 2 days until the termination of the experiment 22 days after implantation. On each day of treatment caliper measurements were taken of the tumours, and transferred direct to mm. squared paper; in this way it was an easy matter to measure the cross-sectional area of tumours of irregular shape. At the end of the experiment, size, *i.e.* cross-sectional area, was estimated, and from the tabulated results the mean was calculated for each group showing the increase during the experimental period. The ratio of the mean values on the 10th and 22nd days respectively was taken as an index of the rate of growth.

Exp. II. In this experiment the tumours used for implantation were known to be of lowered vitality (they were derived from tumours which had recurred after removal, and when implanted into fresh hosts had been found to exhibit a slow rate of growth, and a reduction in the usual percentage of "takes").

Two amino-acids were tested in this experiment for their ability to restore the vitality of this debilitated tissue. Treatment was begun 2 days before implantation, and continued every second day as in Exp. I, and caliper measurements were taken at the same intervals as before.

Special precautions were taken to ensure the uniformity of the implanted tissue in its distribution among the various groups, the following procedure being adopted; the mice were divided into

[1] Supplied by Messrs British Drug Houses, Ltd.

three main groups, two treated, and one control, and each of these was subdivided into four small groups, *A*, *B*, *C*, *D*. One tumour was used to implant all the "*A*" groups, another for *B*, etc. In addition to this the mice were implanted in strict rotation as follows: mouse 1, Group I *A*; mouse 1, Group II *A*; mouse 1, Group III *A*; then mouse 2 in each group as in the first round.

As an extra check upon the usual measurements all the tumours were weighed at the close of the experiment, the mean weight was calculated, and the ratio of each treated group to the control was compared with the ratio obtained by measurement of cross-section. The results will be found in Table III, Exp. II.

The results of these two experiments made it appear almost certain that arginine had a definite effect upon the rate of tumour growth; the variation in the growth potentiality of apparently healthy tumours observed in earlier work, however, had made it desirable to limit the number of animals used in each experiment, in order that the implanted tissue should be derived, as far as possible, from the same source. Consequently it seemed advisable to apply to the results the method described by Fisher [1928] for the determination of the significance of the difference of the means of small samples; the tabulated results therefore include the "*t*" and "*P*" values used in this method. According to Fisher [1928] we may "draw a conventional line at 0·05," *i.e.* if the value is at, or below, this figure the difference may be held to be significant, and would not have occurred by chance, and one may place "a high degree of confidence in the results" if $P = 0{\cdot}01$.

The choice of amino-acids was limited in accordance with the degree of their solubility in water. The low solubility of tyrosine prohibited its use for hypodermic injection; a considerable mortality attended the use of cystine for the same reason, and cysteine was substituted as a result. It was obviously impossible to test the guanidine radical of arginine separately [Barger, 1914], but sodium valerate was included in the series. Glutamic acid was given a separate trial in view of it being a constituent of glutathione; lysine was not tested.

An example of experimental data from which the results were calculated.

(Treatment begun 10 days after implantation of M 63.) Surface area of tumours as estimated by caliper measurements in mm.^{2}

Arginine:	Days since implantation						Ratio of size,
	10	12	14	16	18	22	day 10 : 22
1	16	64	106	154	250	527	1 : 33·0
2	60	118	193	290	332	614	1 : 10·2
3	64	126	166	274	443	714	1 : 11·1
4	96	119	205	254	254	419	1 : 4·3
5	96	156	198	212	260	303	1 : 33·1
6	10	42	58	173	237	430	1 : 43·0
7	23	57	105	175	207	242	1 : 10·5
8	8	24	75	175	260	353	1 : 44·7
9	7	28	36	96	143	216	1 : 30·8
10	4	6	74	131	176	246	1 : 61·5
11	6	6	131	180	210	437	1 : 72·9
12	6	8	137	183	225	257	1 : 32·1
Mean	33·0	62·8	123·6	174·7	249·0	396·0	1 : 29·7

After estimation of the probable error the mean values were respectively $33{\cdot}0 \pm 6{\cdot}6$, and $396{\cdot}0 \pm 29{\cdot}5$.

Controls:	Days since implantation						Ratio of size, day 10 : 22
	10	12	14	16	18	22	
1	11	17	32	32	57	80	1 : 7·3
2	44	66	102	137	196	288	1 : 6·5
3	56	79	121	180	197	257	1 : 4·9
4	5	15	19	39	40	49	1 : 9·9
5	33	67	79	102	159	179	1 : 5·4
6	50	66	99	217	372	497	1 : 9·9
7	13	48	57	82	114	184	1 : 14·1
8	13	16	31	58	74	157	1 : 12·2
9	7	21	21	33	84	265	1 : 37·8
10	47	82	113	113	119	277	1 : 6·0
11	14	24	31	40	51	84	1 : 6·0
12	60	143	171	291	291	336	1 : 5·6
Mean	29·0	46·2	69·8	100·3	146·1	221·0	1 : 10·4

After estimation of the probable error the mean values were respectively $29{\cdot}0 \pm 4{\cdot}4$ and $221{\cdot}0 \pm 24{\cdot}4$.

Difference of mean day 10 = $4{\cdot}0 \pm 0{\cdot}3$.
Difference of mean day 22 = $175{\cdot}0 \pm 3{\cdot}2$.
The value of t on day 10 = 0·3.
The value of P on day 10 = 0·9.
The value of t on day 22 = 3·2.
The value of P on day 22 = 0·01.

Experiment I.

Table I.

Group	No. in group	Size on 10th day: Difference of mean	t	P	Size on 22nd day: Difference of mean	t	P
Arginine Untreated	12 12	$+ 4{\cdot}0 \pm 0{\cdot}3$	0·30	0·90	$+175 \pm 3{\cdot}2$	3·2	0·01
Glycine Untreated	10 7	$\pm 0{\cdot}0$	0·30	0·70	$- 28 \pm 0{\cdot}6$	0·7	0·50
Alanine Untreated	12 7	$+12{\cdot}0 \pm 1{\cdot}3$	1·00	0·30	$+ 5 \pm 0{\cdot}09$	0·1	1·00
Cystine Untreated	6 7	$\pm 0{\cdot}0$	1·00	0·30	$- 25 \pm 0{\cdot}3$	1·4	0·20
Glutamic acid Untreated	10 7	$+ 2{\cdot}0 \pm 0{\cdot}7$	0·60	1·00	$- 14 \pm 4{\cdot}9$	0·2	1·00
Arginine Untreated	4 7	$+ 5{\cdot}0 \pm 0{\cdot}7$	0·14	0·90	$+192 \pm 4{\cdot}2$	3·4	0·01
Sodium valerate Untreated	11 6	$- 8{\cdot}0 \pm 4{\cdot}1$	0·40	0·70	$+ 54 \pm 0{\cdot}5$	0·6	0·50
Arginine Untreated	4 6	$+23{\cdot}0 \pm 0{\cdot}7$	0·90	0·40	$+122 \pm 2{\cdot}1$	1·6	0·10

The comparison of arginine and cystine is of value in view of the importance which has been attached to glutathione in connection with tumour growth. From the results obtained it would appear that neither cystine nor glutamic acid, a constituent of glutathione, has any appreciable effect upon the rate of tumour growth.

The results of the second experiment showed that histidine could not replace arginine as a stimulant of growth. It appeared to exert a certain effect upon the percentage of successful implantations, when tested on devitalised tissue, the number of failures being greater in the case of arginine than histidine, but

Table II. *Ratio of surface area of tumours ten and twenty-two days after implantation.*

Group	Ratio. Group mean	Difference of mean	P
Arginine Untreated	29·7 ± 14·3 10·4 ± 6·0	+19·5 ± 0·84	0·01
Glycine Untreated	17·9 ± 6·8 20·2 ± 6·3	− 2·3 ± 0·17	0·70
Alanine Untreated	15·1 ± 7·1 20·2 ± 6·3	− 5·1 ± 0·36	0·40
Cystine Untreated	21·4 ± 4·4′	+ 1·2 ± 0·63	0·90
Glutamic acid Untreated	19·4 ± 7·5 20·2 ± 6·3	+ 0·8 ± 0·05	1·00
Sodium valerate Untreated	9·8 ± 2·2 11·8 ± 3·5	+ 3·0 ± 0·11	0·15
Arginine Untreated	15·0 ± 7·8 11·8 ± 3·5	+ 8·2 ± 0·07	0·15
Arginine Untreated	52·9 ± 5·7 20·2 ± 6·3	+32·0 ± 2·60	0·01

Table III. *Comparison of the effect of arginine and cystine, or cysteine, the latter being substituted for untreated controls.*

		Size on 10th day			Size on 22nd day		
Group	No. in group	Difference of mean	t	P	Difference of mean	t	P
Arginine Cystine	4 6	±0·0	0·2	0·80	+217 ± 3·1	2·50	0·04
Arginine Cysteine	8	+1·0 ± 0·16	0·1	0·80	+ 49 ± 0·8	0·90	0·35

less in arginine-treated mice than in the untreated controls. The exact percentages of failures were, arginine 37·5 %, histidine 26 %, untreated 50 %. These figures are very high for implantation failures in the tumour M 63.

Experiment II.

Table I.

		Size on 10th day			Size on 22nd day		
Group	No. in group	Difference of mean	t	P	Difference of mean		P
Arginine Untreated	15 12	+14·0 ± 0·4	0·1	0·40	+134·0 ± 4·3	0·5	0·05
Histidine Untreated	17 12	+18·0 ± 0·1	0·1	0·10	+ 38·0 ± 0·8	1·7	0·10

Table II.

Group	No. in group	Ratio. Group mean	Difference of mean	P
Arginine Untreated	15 12	10·5 ± 7·47	5·5 ± 0·06	0·10
Histidine Untreated	17 12	6·8 ± 4·7	1·8 ± 0·06	0·40

Table III.

Group	No. in group	Mean wt.	Difference of mean	t	P
Arginine	15	$1{\cdot}6 \pm 0{\cdot}27$	$0{\cdot}9 \pm 0{\cdot}21$	1·5	0·1
Untreated	12	$0{\cdot}7 \pm 0{\cdot}22$			
Histidine	17	$0{\cdot}7 \pm 0{\cdot}14$	0·0	0·1	0·9
Untreated	12	$0{\cdot}7 \pm 0{\cdot}22$			

Discussion.

From the tabulated results of Experiments I and II it can be seen that among the amino-acids tested for their effect upon tumour growth arginine alone induces a significant difference from the mean of the untreated control group on the 22nd day, as estimated by the value of P.

It is evident that if the size of tumours in a treated group exceeds that of the corresponding untreated group on the 10th day the former would be expected to exceed the latter on the 22nd day as well, apart from any treatment which the animals had received. In certain cases it may therefore appear that the difference of mean in the arginine-treated groups on the 22nd day was not so significant as it otherwise would seem. In order to assess the real value of the difference two points must be taken into consideration, of which one is the value of P. On the 10th day the P value indicates in every case that the difference in mean could be accounted for as the result of random sampling from a homogeneous population. The P value for the 22nd day, on the other hand, in three cases out of four of the arginine-treated groups is 0·05 or less, while the value for the groups treated with other amino-acids is in no case less than 0·1. The second point to be considered is the ratio of the sizes (cross-sectional area), on the 10th and 22nd days respectively within one and the same group. The mean ratio obtained after calculating the individual ratios in such a group can then be compared with its untreated control. The ratio will obviously be greater or less according to the mean size on the 10th day of the experiment unless the mean on the 22nd day is proportionately very great; since in some of the arginine-treated groups the mean was higher on the 10th day than it was in the controls the ratio might have been thus reduced, but in spite of this fact a markedly higher ratio occurs in the arginine-treated groups alone. The second experiment indicates that arginine and histidine have some slight effect in raising the percentage of "takes" in the case of debilitated tissue (as previously defined), but that only arginine has any appreciable influence upon its subsequent rate of growth. The difference in weight in these groups is not significant if judged by the value of P, but the mean weight is about 50 % greater in the arginine-treated group than in the untreated control, which agrees well with the difference in ratio where cross-sectional area was the criterion of size.

From this analysis of the data it seems justifiable to conclude that the influence of arginine upon tumour growth is real, and that it is specific in so far as a similar effect was not observed in the case of any other substance used in

this series of experiments. It is suggested that an increase in nuclear substance leading to an acceleration of the rate of cell division, may be the mechanism through which the observed increase in size is attained. Certain inferences concerning the biology of tumour growth suggest themselves, assuming this hypothesis to be correct, but the consideration of theoretical implications is beyond the scope of the present paper, and the facts are therefore recorded alone.

SUMMARY.

A comparison of the effects upon tumour growth of certain amino- and fatty-acids (arginine, histidine, glycine, alanine, cystine, cysteine, glutamic acid, and sodium valerate), showed that arginine was the only substance investigated which exerted a definite effect upon the rate of tumour growth.

In an experiment dealing with the implantation of "devitalised" tissue it was found that histidine appeared to increase the percentage of "takes." It had no effect upon the subsequent rate of growth, whereas arginine had a definitely stimulating effect.

I wish to express my thanks to Prof. Crew for the facilities provided for the prosecution of this research, and for much helpful criticism and advice, and I would also acknowledge my indebtedness to Dr Kermack, of the Royal College of Physicians, for his advice in the statistical treatment of the results.

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LXVII. A STUDY OF THE ACID-BASE EQUILIBRIUM OF THE OCULAR HUMOURS.

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So long as the fluids in the eye were regarded as the secretory product of the ciliary body no direct relationship between them and the blood stream was postulated. A large amount of experimental work, however, has recently been brought forward which tends to show that this fluid is a dialysate of the capillary plasma [see Duke-Elder, 1927, 1], and if this theory is correct the relationship between the intra-ocular fluid and the blood-plasma should be very intimate. Most of the evidence which has been brought forward in support of the newer theory is either chemical in nature, dealing with the constitution of normal and abnormal aqueous humour, or physical, dealing with the hydrostatic, osmotic and electrostatic forces involved. It may be of value therefore to consider how far the relationship of the acid-base equilibrium of the fluids in the eye with that of the plasma brings support to the more recent conceptions.

It was considered desirable to estimate the hydrogen ion concentration of the vitreous and aqueous humours, the carbon dioxide content of the aqueous humour, and the carbon dioxide content and capacity as well as the dissolved oxygen in the vitreous humour. The eyes were obtained immediately after death and the aqueous humour was abstracted by means of a syringe and kept under paraffin oil. The vitreous humour was then shelled out through a posterior incision and was kept in a paraffin-covered tube.

Since the vitreous humour is difficult to measure accurately on account of its jelly-like consistency, it was used after liquefaction by forcing it through the perforated plunger of a syringe the opening of which had been plugged.

p_H DETERMINATION.

Cullen's [1922] colorimetric method as applied in Myers's [1924] technique was employed. Since both humours are only slightly buffered in comparison with the blood-plasma, the p_H determinations were carried out without any dilution. The values are only comparative in respect to the two humours, since no corrections were made for temperature (Tables I and II).

Table I. *Aqueous humour.*

p_H *values of the aqueous humours of cattle varying in age from 5 weeks to 2 years.*

No. of animal	Age of animal	Room temp.	p_H Eye 1	p_H Eye 2	p_H Average
1	5 wks.	26°	7·20	7·25	7·23
2	2 mths.	27	Mixture		7·22
3	4 ,,	30	7·43	7·33	7·38
4	4 ,,	27·5	7·40	—	7·40
5	4 ,,	27	7·27	7·32	7·30
6	4 ,,	27	Mixture		7·41
7	8 ,,	27	7·33	7·44	7·39
8	1½ yrs.	24	7·42	7·44	7·43
9	1½ ,,	24	7·40	7·41	7·41
10	1½ ,,	24	7·38	—	7·38
11	1½ ,,	25·5	*7·35	7·35	7·35
12	1½ ,,	25·5	*7·10	7·20	7·15
13	1½ ,,	25·5	*7·35	7·25	7·30
14	1½ ,,	25·5	*7·42	7·36	7·39
15	1½ ,,	26	*7·25	7·20	7·23
16	1½ ,,	26	*7·36	7·20	7·28
17	1½ ,,	26	*7·35	7·33	7·34
18	2 ,,	27·5	7·28	7·26	7·27
19	2 ,,	27·5	7·35	7·34	7·35
20	2 ,,	26	7·31	7·40	7·36
21	2 ,,	26	7·20	—	7·20
22	2 ,,	26	Mixture		7·40
23	2 ,,	26	7·58	7·48	7·53
24	2 ,,	26	7·48	7·58	7·53
25	2 ,,	25·5	7·39	7·44	7·32
26	2 ,,	25·5	7·51	7·42	7·47
27	2 ,,	25·5	7·45	7·45	7·45
28	2 ,,	25	*7·28	7·33	7·31
29	2 ,,	25	*7·20	7·25	7·23
30	2 ,,	25	*7·27	7·25	7·26
31	2 ,,	25	*7·18	—	7·18
32 (Sheep)	Age unknown	27	7·23	—	7·23

* The humours were allowed to remain in the extracted eye in the refrigerator half a day. All other results were obtained from strictly fresh humours.

When the ocular humours are allowed to remain in the excised eyes for some hours, they become more acid (see Tables III and IV), despite a possible loss of carbon dioxide, which may be due to autolytic decomposition of protein. Unpublished earlier analyses by the author (1927), in confirmation of the findings of other workers, revealed that the vitreous humour under these conditions suffers a great increase in protein content. This increase in protein content is thought to be due to transudation from the surrounding capillaries [Duke-Elder, 1927, 2].

A comparison of the average p_H values of aqueous humours of cattle for different age groups with those of vitreous humours for similar age groups shows that the aqueous humour is slightly less alkaline than the vitreous humour. This is illustrated in Fig. 1.

Table II. *Vitreous humour.*

p_H *values of the vitreous humours of cattle varying in age from* 3 *months to* 2 *years.*

No. of animal	Age of animal	Age of specimen under oil (days)	Room temp.	p_H Determinations 1	2	3	Average
1	3 mths.	Fresh	27°	7·27	7·20	7·31	7·26
2	4 ,,	,,	27·5	7·40	7·41	—	7·40
3	8 ,,	,,	27	7·36	7·43	—	7·40
4 Eye 1	1½ yrs.	15	24	*7·36	7·33	—	7·35
Eye 2	1½ ,,	15	24	*7·33	7·31	—	7·32
5	1½ ,,	15	24	*7·30	7·30	7·15	7·30
6	1½ ,,	16	22	*7·14	7·20	7·15	7·16
7	1½ ,,	4	22·5	*7·24	7·24	—	7·24
8 Eye 1	1½ ,,	4	22·5	*7·24	7·24	—	7·24
Eye 2	1½ ,,	4	22·5	*7·25	7·30	—	7·27
9	1½ ,,	3	25	7·45	—	—	7·45
10	1½ ,,	3	25	7·54	—	—	7·54
11	1½ ,,	3	25	7·49	—	—	7·49
12	1½ ,,	3	25	7·44	—	—	7·44
13	1½ ,,	3	25	7·40	7·40	—	7·40
14	1½ ,,	3	25	7·40	—	—	7·40
15	1½ ,,	3	25	7·35	7·36	—	7·36
16 Eye 1	2 ,,	Fresh	25	7·44	7·38	—	7·41
Eye 2	2 ,,	,,	25	7·38	7·42	—	7·40
17 Eye 1	2 ,,	1	24	7·44	7·40	7·45	7·43
Eye 2	2 ,,	1	24	7·40	7·40	7·38	7·39
18 Eye 1	2 ,,	2	26	7·49	7·54	7·54	7·52
Eye 2		2	26	7·49	7·56	7·54	7·53
19	2 ,,	2	26	7·66	7·65	7·65	7·65
20 Eye 1	2 ,,	2	26	7·64	7·58	7·64	7·61
Eye 2		2	26	7·63	7·61	—	7·62
21 Eye 1	2 ,,	6	25·5	*7·49	7·54	—	7·51
Eye 2		6	25·5	*7·24	7·33	7·38	7·32
22 Eye 1	2 ,,	6	26	*7·40	7·43	—	7·41
Eye 2		6	26	*7·32	7·33	—	7·33
23	2 ,,	6	26	*7·46	7·36	—	7·41
		6	26	*7·35	7·40	7·35	7·37

* The humours were allowed to remain in the excised eye for half a day in the refrigerator.

Table III. p_H *values for fresh aqueous humours and for those kept in the excised eye, in the refrigerator, for half a day.* (*The specimens were handled under oil.*)

Fresh aqueous: No. of animal	Fresh aqueous: p_H averages	Aqueous kept in the extracted eye for half a day: No. of animal	Aqueous kept in the extracted eye for half a day: p_H averages
	Animals 6 months old.		
8	7·43	11	7·35
9	7·41	12	7·15
10	7·38	13	7·30
—	—	14	7·39
—	—	15	7·23
—	—	16	7·28
—	—	17	7·34
Average	7·41		7·29
	Animals 2 years old.		
18	7·27	29	7·28
19	7·35	30	7·20
20	7·36	31	7·27
21	7·20	32	7·18
22	7·40	—	—
23	7·53	—	—
24	7·53	—	—
25	7·42	—	—
26	7·47	—	—
27	7·45	—	—
Average	7·40		7·23

Table IV. p_H *values for fresh vitreous humours and those kept in the excised eyes, in the refrigerator, for half a day.* (*All were handled under oil.*)

Vitreous removed immediately		Vitreous kept in the extracted eye for half a day	
No. of animal	p_H averages	No. of animal	p_H averages
	Animals 1½ years old.		
9	7·45	4	7·35
10	7·54	4	7·32
11	7·49	5	7·30
12	7·44	6	7·16
13	7·40	7	7·24
14	7·40	8	7·24
15	7·36	8	7·24
Average	7·44		7·27
	Animals 2 years old.		
16	7·41	21	7·51
16	7·40	21	7·32
17	7·43	22	7·41
17	7·39	22	7·33
18	7·52	23	7·41
18	7·53	23	7·37
19	7·65		
20	7·61		
20	7·62		
Average	7·51		7·39

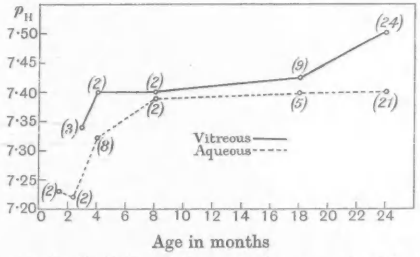

Fig. 1. Rate of increase in alkalinity of the ocular humours of cattle with advancing age. (Figures in parentheses denote the number of eyes on which the averages were based.)

Carbon dioxide determinations.

Analyses for carbon dioxide were made by the method of Van Slyke and Neill [1924], in which 2 cc. of the fluid were used.

The vitreous humour only was studied in respect to the relationship of the carbon dioxide content to the age of the animal. The carbon dioxide content of the vitreous humour decreases with increasing age. This is shown by the arrangement of the data in Table V and particularly by the average values for various ages presented in Table VI.

The aqueous humour was analysed for its carbon dioxide content only (see Table VIII). In the vitreous humour both carbon dioxide content and capacity were studied. The carbon dioxide capacity determination differed from the

Table V. *Carbon dioxide content of the vitreous humours from cattle varying in age from 6 weeks to 2 years.*

No. of animal	Age of animal	No. of eye	Age of specimen under oil (days)	Vol. % CO_2 content: Determinations 1	2	3	Average
1	6 wks.	1	Fresh	58·7	58·5	59·5	58·9
		2	,,	58·1	—	—	58·1
2	2 mths.	Mixture	2	74·1	74·3	—	74·2
3	3 ,,	,,	Fresh	60·8	60·1	—	60·5
4	4 ,,	,,	,,	52·8	52·1	52·0	52·3
5	8 ,,	,,	,,	59·5	59·9	—	59·7
6	1 yr.	,,	,,	54·9	—	—	54·9
7	1 ,,	,,	,,	54·9	—	—	54·9
8	1 ,,	,,	,,	62·3	—	—	62·3
			3	60·9	59·9	—	60·4
			7	60·8	—	—	60·8
9	1½ yrs.	,,	4	46·2	46·5	—	46·4
10	1½ ,,	,,	,,	44·1	46·2	44·7	45·0
11	1½ ,,	,,	,,	41·2	42·2	—	41·7
12	2 yrs.	1	Fresh	51·3	51·5	50·7	51·1
		2	3	52·4	—	—	52·4
13	2 ,,	1	1	45·4	44·3	44·5	44·7
		2	,,	45·1	45·1	—	45·1
14	2 ,,	1	Fresh	46·3	46·7	—	46·5
15	2 ,,	1	,,	46·3	46·0	—	46·2
		2	,,	44·7	—	—	44·7
16	2 ,,	1	,,	45·6	46·4	—	46·0
17	2 ,,	1	,,	46·3	—	—	46·3
		2	,,	47·1	46·3	—	46·7
18	2 ,,	1	15	38·0	37·3	—	37·7
		2	15	37·3	37·3	—	37·3
19	2 ,,	1	15	38·3	—	—	38·3
20	2 ,,	1	16	40·8	40·6	—	40·7
21	2 ,,	1	2	48·5	—	—	48·5
		2	2	54·5	—	—	54·5
22	2 ,,	1	2	55·4	—	—	55·4
23	2 ,,	1	2	43·5	—	—	43·5
24	2 ,,	1	6	42·7	40·8	—	41·8
25	2 ,,	1	6	44·8	—	—	44·8
26	2 ,,	1	6	43·7	—	—	43·7
27	2 ,,	1	6	44·7	—	—	44·7
28	2 ,,	1	6	47·1	—	—	47·1
29	2 ,,	1	Fresh	47·5	47·4	—	47·5
		2	,,	45·7	47·1	—	47·4
30	2 ,,	1	1	43·8	42·3	—	43·1
		2	1	45·2	45·6	—	45·4

Table VI. *Average values of the carbon dioxide content of vitreous humours of different ages.*

Age	6 wks.	2 mths.	3 mths.	4 mths.	8 mths.	1 yr.	1½ yrs.	2 yrs.
Vol. % of CO_2	58·7	74·2	60·5	52·3	59·7	59·0	44·4	42·9
No. of animals used	1	1	1	1	1	3	3	19

carbon dioxide content determination in that in the former analysis the vitreous humour was equilibrated with alveolar air before the carbon dioxide was withdrawn. Under these conditions the vitreous humour took up no additional carbon dioxide. Therefore the carbon dioxide content and capacity of the vitreous humour are alike. The results are given in Table VII.

Table VII. *The carbon dioxide content compared with the carbon dioxide capacity of the vitreous humour.*

No. of animal	Age of animal	Age of specimen	Vol. % CO_2 content: Determinations 1	2	3	Average	Vol. % CO_2 capacity: Determinations 1	2	Average
4	4 mths.	Fresh	52·8	52·1	52·3	52·3	51·7	52·3	52·0
12	2 yrs.	3 days	52·4	—	—	52·4	51·4	51·9	51·7

Table VIII. *Aqueous humour.*

The carbon dioxide content of fresh aqueous humour compared with that previously used in a colorimetric p_H *determination. (The humours in both cases were obtained from cattle* $1\frac{1}{2}$*–2 years old, and handled under oil.)*

No. of animal	Original sample	Vol. % CO_2 content; After p_H determination: Determinations 1	2	3	Average
1	49·2	—	—	—	—
2	49·4	47·8	—	—	47·8
3	47·6	47·5	—	—	47·5
4	48·9	—	—	—	—
5–8	40·3	36·8	—	—	36·8
9–11	—	37·9	38·7	38·0	38·2
12–13	45·1	39·5	39·7	—	39·6
14–16	47·9	—	—	—	—
14	—	38·9	—	—	38·9
15	—	50·4	—	—	50·4
16	—	40·2	—	—	40·2
17	—	42·4	—	—	42·4
17–18	—	40·8	—	—	40·8
19	53·5	—	—	—	—
20	45·7	—	—	—	—
Average	47·5				42·3

Table IX. *Vitreous humour.*

The carbon dioxide content of fresh vitreous humour compared with that of vitreous humour previously used in a colorimetric p_H *determination. (The humours in both cases were obtained from cattle* $1\frac{1}{2}$*–2 years old, and handled under oil.)*

No. of animal	Age of specimen (days)	Vol. % CO_2 content; averages: Original	After p_H det.
21	2	48·5	46·3
21	2	54·5	56·0
22	2	55·4	53·4
23	2	43·5	42·4
24	6	41·8	38·2
25	6	44·8	44·5
26	6	43·7	41·5
27	6	44·6	43·5
27	6	47·1	42·3
	Average	47·1	45·3

Carbon dioxide content was determined on several samples of both humours after p_H determinations had been carried out on them, and the results were compared with those obtained on the same humours in their original fresh state. The results given in Tables VIII and IX show that both aqueous and vitreous humours tend to lose their carbon dioxide, if disturbed, even under oil, the aqueous humour more so than the vitreous humour.

Several determinations for carbon dioxide content of the plasma and the vitreous humour in the same animal show that the carbon dioxide content of the vitreous humour is lower than that of the plasma.

Table X. *The carbon dioxide content of ox-plasma as compared with the carbon dioxide content (or capacity, see Table VII) of the vitreous humour of the same animals. The figures express average values.*

No. of animal	Age of animal (yrs.)	Vol. % CO_2 plasma	Vol. % CO_2 vitreous
12 and 13	2	59·1 (2)*	47·8 (9)*

* This figure indicates the number of determinations on which the average is based.

Comparing the figure 47·5 vol. % obtained on fresh aqueous humour of cattle 1½–2 years old (Table VIII) with 43·7 vol. %, the average figure for carbon dioxide content of fresh vitreous humour of cattle of the same age (Table V), it is seen that the carbon dioxide content of the aqueous exceeds that of the vitreous humour by 3·8 vol. %. Hence the order of increasing values of carbon dioxide content is represented in the ocular humours and the blood-plasma of cattle of the same age, as follows: vitreous, 43·7 vol. %; aqueous 47·5 vol. %; plasma 59·1 vol. %.

Table XI. *The carbon dioxide content of fresh vitreous humour as compared with vitreous preserved in the refrigerator under and without oil.*

		Vol. % CO_2 content; averages		
No. of animal	Age of animal (yrs.)	Fresh	Preserved under oil	Days in refrigerator
8	1	62·3	60·5	3–7
12	2	51·1	52·4	3
			Preserved without oil	
14	2	46·5	46·7	3–7

The carbon dioxide content of the vitreous humour was studied also after the humour had been kept in the refrigerator for several days, both without oil and under oil. The figures presented in Table V, and the results shown in Table XI, indicate that the vitreous humour, whether under oil or exposed, can be kept in the refrigerator for about a week without loss of carbon dioxide. The average of the nine determinations made on the fresh vitreous humours from 2-year old animals in Table V is 46·9 vol. %. The average of the fourteen determinations on similar vitreous humours that had been kept in the refrigerator from one to six days is 46·1 vol. % (Table V). A detectable loss of

carbon dioxide occurs, however, when the humour, even under oil, is allowed to stand for longer than a week, as shown by the results on animals 18, 19 and 20 in Table V.

Oxygen determinations on the vitreous humour.

The analyses were made by the Van Slyke and Neill [1924] method, in which 2 cc. of the fluid were employed.

It was thought probable that the vitreous humour would contain little, if any, oxygen, except the traces in physical solution. Table XII proves this conjecture to be correct.

Table XII. *Analysis of the vitreous humour for dissolved oxygen.*

No. of animal	No. of determinations	Mm. Hg before absorption of O_2	Mm. Hg after addition of $Na_2S_2O_4$	Difference in mm. Hg
1	1	116·5	116·2	+0·3
1	2	114·0	116·5	−2·5
1	3	109·0	110·8	−1·8
1	4	108·5	111·0	−2·5
2	5	115·0	118·0	−3·0

A blank determination gave a figure of 0·5 mm. Hg. When this figure is subtracted from those of the last column, a negative value is obtained for oxygen in each case. Any amount of oxygen larger than the equivalent of experimental error would be represented under the assumed conditions by a positive value. The fact that all the five values happen to be negative suggests that some other gas was evolved on addition of $Na_2S_2O_4$ and proves (assuming the amount of this gas to be negligible) that the vitreous humour contains no dissolved oxygen. The gas evolved was identified, by its odour, as sulphur dioxide.

Discussion.

The carbon dioxide content in order of decreasing values is represented by the fluids of cattle 1½–2 years old as follows: plasma, 59·1 vol. %; aqueous humour, 47·5 vol. %; vitreous humour, 43·7 vol. %.

No direct experimental data are available which would show whether changes in the hydrogen ion concentration of the blood are reflected in the ocular humours as shown in respect to sugar and carbon dioxide. The present work brings out the fact that the vitreous humour is slightly more alkaline than the aqueous humour. This is to be explained from the fact that the vitreous humour contains at least twice as much protein as the aqueous humour. Duke-Elder [1927, 3] has shown that the aqueous humour is particularly rich in the acid radicals (Cl^-, $PO_4^{\equiv}$, $SO_4^{=}$) of the inorganic salts present in the body fluids, whereas the blood-plasma exceeds the aqueous humour in respect to the cations (Na^+, K^+, Ca^{++}, Mg^{++}) of the same salts. It is an accepted fact that the cations are held more or less in combination by the blood-proteins, and thus partially withdrawn from body fluids that contain less protein toward

those that contain more. It is to be expected therefore that the plasma, since it contains more protein than the vitreous humour, must be more alkaline than the latter. According to data obtained by Baurmann [1927], the p_H values of human aqueous humours vary between 7·20 and 7·35, with an average of 7·27. These figures are definitely lower than those for blood, which are 7·35 to 7·50. The data of the present work show, on the whole, smaller differences between the aqueous and vitreous humours of cattle than Baurmann observed between plasma and aqueous humour in man. It is clear that the order of increasing values of the hydrogen ion concentration (or decreasing alkalinity) on the p_H scale is represented by the ocular humours and the plasma as follows: p_H of plasma $>$ p_H of vitreous humour $>$ p_H of aqueous humour.

In view of the foregoing, there is sufficient reason to postulate that changes in the hydrogen ion concentration of the blood would be readily reflected in the ocular humours, particularly in the aqueous humour.

Summary.

(1) Carbon dioxide content and capacity are identical for the vitreous humour. The amount decreases, with a few fluctuations, on the average from 58·7 vol. % in a calf 6 weeks old to 42·9 vol. % in cattle 2 years old. Plasma exceeds the vitreous humour in carbon dioxide capacity by an average of 11 vol. % in 2-year old cattle. The vitreous humour, in its natural state, can be kept under oil in the refrigerator for about 1 week without loss of carbon dioxide. If, however, its structure is destroyed by mechanical liquefaction, it tends to lose its carbon dioxide much more readily.

(2) No dissolved oxygen could be demonstrated in the vitreous humour.

(3) The aqueous humour of cattle $1\frac{1}{2}$–2 years old contained an average of 47·5 vol. % carbon dioxide. On the other hand, an average figure of 42·3 vol. % was obtained on the same or similar humours after they had been used in a colorimetric p_H determination. Hence, the aqueous humour loses its carbon dioxide very readily if disturbed, even under oil.

(4) The carbon dioxide content of the aqueous humour exceeds that of the vitreous by 3·8 vol. % for cattle $1\frac{1}{2}$–2 years old, the average figures being 47·5 vol. % and 43·7 vol. % respectively.

(5) There is a distinct decrease in the hydrogen ion concentration of the aqueous humour of cattle with advancing age. The relative p_H values increase from 7·23 in a 5-week old calf to 7·40 in 2-year old cattle. (No correction was made for temperature.)

(6) The relative p_H values of the vitreous humour of cattle increase, on the average, from 7·29 in 3-month old calves to 7·50 in 2-year old cattle.

(7) The aqueous humour is slightly, but consistently, more acid than the vitreous humour.

My thanks are due to Dr C. S. O'Brien, Dr H. A. Mattill, and Dr C. W. Rutherford for their valuable help and advice. I am also indebted to Dr V. C. Myers for suggesting the work.

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LXVIII. THE INACTIVATION OF TRYPSIN BY HEAT.

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The study of the heat-inactivation of trypsin contained in a crude extract of dried pancreas gland powder is complicated by the "spontaneous activation" which occurs in such extracts. Waldschmidt-Leitz and Harteneck [1925] have recently interpreted this phenomenon as due to the production of enterokinase from its pre-stage, which occurs together with the trypsin in the pancreas. This transformation takes place in aqueous extracts of dried pancreas powder, or in the dried powder itself on prolonged storage. As a consequence, in an aqueous extract of dried pancreas powder maintained at constant temperature two processes occur, heat-inactivation of the trypsin and simultaneously "spontaneous activation" of the enzyme. Thus, if the trypsin content of the extract be measured from time to time by hydrolysis of a suitable protein, there may be, instead of the decrease to be expected from heat-inactivation of the enzyme, an apparent increase in trypsin content, if it so happens that the "spontaneous activation" effect preponderates over the heat inactivation. Furthermore, Waldschmidt-Leitz has indicated that trypsin associated with its activator is less stable than the enzyme alone. In view of this the main experiments described in this paper have been carried out with a purified extract of the dried pancreas powder, in which the trypsin is free from enterokinase and its pre-stage.

Experimental procedure.

To study the inactivation of trypsin at different p_H values the following procedure was adopted. To 10 cc. of the neutral solution prepared in the manner described below were added normal acetic acid or normal ammonia to bring it to a required p_H and then distilled water was added to make the total volume 11 cc. This volume of solution was divided out into a number of test-tubes already immersed in the thermostat, regulated to $\pm 0{\cdot}05°$, at 50°. The test-tubes were then tightly stoppered and removed at various time intervals from the thermostat into crushed ice. The trypsin content of 1 cc. of solution was then measured. For the major portion of the work the trypsin content was measured by the method of Willstätter *et al.* [1926] (activation

with enterokinase and subsequent interaction with caseinogen at p_H 8·7 at 30° for ~~2~~ 20 minutes). While the work was in progress a modification of this method was published by Linderstrøm-Lang and Steenberg [1929]. (This modification provides for a more definite control of the activation process with enterokinase in the analytical procedure. A buffer is used and the activation is always carried out with an arbitrarily chosen "enterokinase unit." Further, the hydrolysis is made to take place at p_H 9·1.) Accordingly the measurements, already made by the writer for the determination of the critical increment of the inactivation process, were repeated using this revised technique and substantially the same results were obtained.

Preparations.

(*a*) *Preparation of trypsin free from enterokinase and the pre-stage of the activator.* In the earlier work of the Willstätter school a separation of trypsin from the activator and its pre-stage was accomplished by a series of selective adsorptions on alumina C_γ. Later a revised method and technique was published by Waldschmidt-Leitz and Linderstrøm-Lang [1927]. The principle of the latter method is to adsorb trypsin-kinase and the pre-stage of the activator on caseinogen, and then, by precipitating the protein, to leave the trypsin behind in the mother liquor. This process of purification, due to Waldschmidt-Leitz and Linderstrøm-Lang was carried out as follows.

Pig's pancreas, free from fat, was prepared by drying with acetone and ether according to the procedure of Willstätter and Waldschmidt-Leitz [1923]. 6 g. of the dried gland powder were added to 100 cc. of glycerol-water mixture (containing 80 % glycerol). The powder was dispersed through the liquid by shaking and the dispersion kept for 5 hours at 30°. The finely divided suspension was centrifuged, and the supernatant liquid finally filtered. To 25 cc. of the clear ice-cold glycerol extract prepared in this manner were added 15 cc. of an ice-cold 6 % caseinogen solution. The mixture was left for 5 minutes in the ice-chest and was then treated with 2 cc. of *N* acetic acid. The resulting precipitate of protein was filtered off and to 20 cc. of the filtrate was added a further 15 cc. of ice-cold 6 % caseinogen solution followed by 1 cc. of *N* acetic acid. The precipitate was again filtered off. The filtrate, now clear and colourless, was brought to neutrality by addition of *N* ammonia. This neutral solution, which contains the trypsin completely free[1] from enterokinase and its pre-stage, was kept in the ice-chest.

(*b*) *Enterokinase solution.* Pig's intestinal mucosa was treated with acetone, acetone-ether mixture, and finally ether according to the procedure of Waldschmidt-Leitz [1924]. An aqueous extract was prepared by suspending 6 g. of the dried mucosa in 200 cc. distilled water for 5 hours at 30°. The suspension was centrifuged and filtered. To 100 cc. of the clear filtrate were

[1] This statement is borne out by the fact that there was no hydrolysis of caseinogen in 2 hours at 30°.

added 1·6 cc. *N* acetic acid and the resulting precipitate of protein matter was centrifuged off. The clear supernatant liquid was neutralised with *N* ammonia and kept in the ice-chest.

(*c*) *Caseinogen solution.* The caseinogen solution was prepared by addition of 100 cc. 0·05 *N* ammonia to 6 g. caseinogen (Kahlbaum-Hammarsten), stirring and keeping for 1 hour at 30°. This solution is referred to as a 6 % caseinogen solution.

(*d*) *Buffer solutions.* For the method of determination according to Willstätter *et al.* [1926] the buffer solution was made up by mixing equal volumes of *N* ammonia and *N* ammonium chloride. The p_H of this buffer was measured at 20° with the glass electrode and found to be 8·6.

For the Linderstrøm-Lang and Steenberg modification of the method two buffer solutions were made up as follows:

(i) by mixing 300 cc. *N* ammonium chloride and 50 cc. *N* ammonia;

(ii) by mixing 250 cc. *N* ammonia and 350 cc. water.

The p_H measurements of the trypsin solution were made by means of the glass electrode at room temperature.

The course of the reaction.

The heat-inactivation of a number of enzymes has been studied by various investigators and in general it has been found that the course of the reaction is "unimolecular." Thus Tammann [1895] found that the heat-inactivation of emulsin conforms to the unimolecular expression, and Madsen and Walbum [1907] obtained the same result for the proteolytic enzymes pepsin and trypsin.

It should be emphasised that all previous work on the heat-inactivation of trypsin has been carried out with material in which the enzyme has been associated with the activator, enterokinase. Since trypsin associated with the activator, *i.e.* trypsin-kinase, and non-activated trypsin are quite specific in their function as catalysts, it cannot reasonably be assumed that the loss of their catalytic efficiency on heating will occur at the same rate. Consequently previous work on the enzyme is not strictly comparable with the results described in this paper obtained with purified trypsin solution. Furthermore, most of the previous work has been carried out with unpurified material containing a considerable amount of protein matter. In such enzyme material, which contains the trypsin associated with enterokinase and therefore endowed with the capacity to hydrolyse native proteins, it is to be expected that hydrolysis of the protein matter contained in the enzyme preparation will proceed during the course of the inactivation. Thus at least two factors are introduced which may influence the temperature sensitivity of the enzyme, namely:

(1) a change in p_H, due to the formation of free carboxyl and amino-groups;

(2) the formation of reaction products which have a stabilising influence upon the enzyme, as the work of Schmidt [1911], Vernon [1904] and Northrop [1922] has shown.

Although the results obtained by Tammann and by Madsen and Walbum were in agreement with the unimolecular equation this is not the experience of other workers. Thus Northrop [1922], working with "Fairchild's trypsin," found that the course of the heat-inactivation did not conform to the unimolecular expression. He suggested that the observed decrease in the unimolecular constants with time is due to the enzyme acting upon the protein matter in the preparation used and thus forming a compound with the products of reaction which is more stable to heat than the "free" enzyme. On purifying the enzyme solution by dialysis he obtained, in one experiment, a satisfactory unimolecular constant. Vernon [1904], working with a pancreas extract containing activator, had also previously found that the constants worked out on the unimolecular basis decreased with time. He ascribed this falling off as due to the presence of several enzymes of different degrees of stability. In the light of the more recent work it is difficult to accept this explanation, for Vernon measured the quantity of enzyme by the hydrolysis of fibrin, and therefore was actually measuring the quantity of one enzyme only, namely activated trypsin, since non-activated trypsin and erepsin do not possess the property of hydrolysing native proteins.

In the experiments described in this paper, with trypsin freed from enterokinase and its pre-stage, it is found that the course of the heat-inactivation in fact follows the unimolecular expression. It would appear, as Northrop suggested, that the decrease in the unimolecular constants observed with unpurified trypsin solutions is due to the protective influence of the substances formed by the action of trypsin-kinase upon the protein in the solution.

The applicability of the unimolecular expression for the heat-inactivation of the trypsin solution employed by the writer is illustrated in Table I, for solutions at p_H values in the alkaline, acid and neutrality regions respectively.

The measurements at a particular time interval were done in duplicate and in all the following tables the magnitude of the "trypsin unit per cc." set down opposite a particular time is the mean of such duplicate experiments.

The values of k_{uni} were obtained from the equation:

$$k_{uni} = \frac{2{\cdot}303}{t} \log_{10} \frac{a}{a-x}$$

where a = trypsin units per cc. at zero time,

$a - x$ = trypsin units per cc. after time t mins.

The trypsin unit employed is that defined by Willstätter *et al.* [1926], namely the quantity of trypsin which produces in a 6 % caseinogen solution under the standard conditions of measurement an increase in acidity equivalent to 1·05 cc. 0·2 *N* NaOH.

Table I.

Temp. 50°. Glycerol content of solution 24 %.

Time t mins.	Trypsin units per cc.	k_{uni}
	p_H 8·40.	
0	1·60	—
60	1·25	$3·97 \times 10^{-3}$
129	1·05	$3·27 \times 10^{-3}$
161	0·905	$3·54 \times 10^{-3}$
211	0·78	$3·40 \times 10^{-3}$
	p_H 5·86.	
0	1·52	—
60	1·28	$2·86 \times 10^{-3}$
124	1·07	$2·82 \times 10^{-3}$
159	0·94	$3·02 \times 10^{-3}$
	p_H 7·12.	
0	1·60	—
120	1·22	$2·26 \times 10^{-3}$
240	0·93	$2·24 \times 10^{-3}$
360	0·77	$2·04 \times 10^{-3}$

THE EFFECT OF p_H UPON THE HEAT INACTIVATION.

The p_H of the medium has a very marked effect upon the stability of all enzymes. In general it is found that each enzyme has a fairly broad optimum region of stability and that outside this region the rate of destruction increases rapidly. In Table II the results obtained for the heat-inactivation of trypsin at various p_H values are summarised. The mean of the values for k_{uni} at a particular p_H are set down opposite that p_H value.

These results are plotted in Fig. 1, where it is seen that the enzyme is most stable at p_H 6·5.

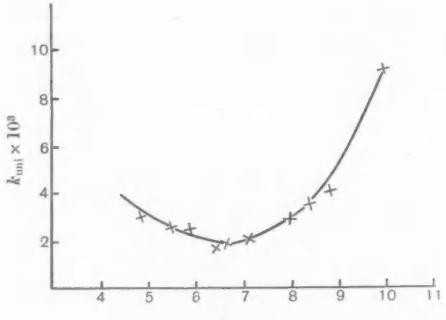

Fig. 1.

Table II.

Temp. 50°. Glycerol content of solution 24 %.

p_H	$k_{uni} \times 10^3$	p_H	$k_{uni} \times 10^3$
9·95	9·23	6·64	2·00
8·82	4·12	6·51	1·70
8·40	3·59	5·86	2·90
7·96	2·98	5·52	2·64
7·12	2·18	4·85	3·14

It is not possible to compare with certainty these results obtained for the effect of p_H with other work in which a different enzyme material has been employed. This point has been emphasised by Willstätter, Haurowitz and Memmen [1924] for the dependence of enzyme activity upon p_H, where they show that substances of various kinds may modify the influence of p_H. At the same time it is noteworthy that for many enzymes the region of optimum stability appears to lie about p_H 6. Thus Morgulis and Beber [1928] found that the optimum stability for catalase lies between p_H 6 and 6·5, Michaelis and Rothstein [1922] found the optimum stability for pepsin at p_H 6, whilst Ernstrom [1922] found an optimum stability at p_H 6·1 for salivary amylase, and Willstätter, Graser and Kuhn [1922] found the optimum stability of saccharase at p_H 5·8. Northrop [1922] found the optimum stability for the trypsin he used to be at p_H 5·0.

The shape of the p_H-stability curve is somewhat similar to that obtained by Lewis [1926] for the effect of p_H upon the rate of heat-denaturation of proteins.

The critical increment of the process.

The critical increment for the process of heat-inactivation of enzymes is found to be very large and comparable with that found for the heat-denaturation of proteins by Chick and Martin [1910] and Lewis [1926].

Experiments were carried out to determine the critical increment for the heat-inactivation of trypsin. This was determined by measuring k_{uni} at 50° and 60° and then substituting the mean values in the equation:

$$\frac{d \ln k_{uni}}{dT} = \frac{E}{RT^2}.$$

Measurements were made in the three cases:

(1) at the region of optimum stability, p_H 6·51;

(2) at an alkaline p_H, 8·82;

(3) at an acid p_H, 5·02.

The results are set out in the following tables, where the method employed for the determination of the quantity of enzyme is also stated.

Table III. *The critical increment at* p_H *6·51.*

Method of determination that of Willstätter *et al.* [1926]. Glycerol content of solution 24 %.

Time t mins.	Trypsin units per cc.	k_{uni}
	Temp. 50°.	
0	1·53	—
120	1·23	$1·82 \times 10^{-3}$
295	0·95	$1·60 \times 10^{-3}$
385	0·80	$1·68 \times 10^{-3}$
	Mean	$1·70 \times 10^{-3}$
	Temp. 60°.	
0	1·51	—
18	1·23	$1·14 \times 10^{-2}$
36	1·03	$1·06 \times 10^{-2}$
54	0·805	$1·16 \times 10^{-2}$
72	0·62	$1·24 \times 10^{-2}$
	Mean	$1·15 \times 10^{-2}$

Substituting the mean values of k_{uni} at 50° and 60° into the equation cited above we arrive at a value of $E = 40{,}800$ calories for the heat-inactivation of trypsin at p_H 6·51.

Table IV. *The critical increment at* p_H *6·50.*

Method of determination that of Linderstrøm-Lang and Steenberg [1929]. Glycerol content of solution 24 %.

Time t mins.	Trypsin units per cc.	k_{uni}
	Temp. 50°.	
0	0·72	—
90	0·60	$2·01 \times 10^{-3}$
160	0·55	$1·68 \times 10^{-3}$
200	0·49	$1·92 \times 10^{-3}$
	Mean	$1·87 \times 10^{-3}$
	Temp. 60°.	
0	0·72	—
20	0·56	$1·25 \times 10^{-2}$
40	0·45	$1·17 \times 10^{-2}$
60	0·35	$1·20 \times 10^{-2}$
	Mean	$1·21 \times 10^{-2}$

From these values of k_{uni} at 50° and 60° we arrive at a value of $E = 39{,}700$ calories for p_H 6·50.

Table V. *The critical increment at* p_H *8·2.*

Method of determination of enzyme quantity that of Willstätter *et al.* [1926]. Glycerol content of solution 24 %.

Temp. 50°. Mean value of $k_{uni} = 4·35 \times 10^{-3}$
Temp. 60°. Mean value of $k_{uni} = 3·47 \times 10^{-2}$

From these values of k_{uni} at 50° and 60° we arrive at a value of 44,300 calories for the critical increment at p_H 8·82.

Table VI. *The critical increment at* p_H *5·02.*

Method of determination of enzyme quantity that of Linderstrøm-Lang and Steenberg [1929].

Temp. 50°. Mean value of $k_{uni} = 2·90 \times 10^{-3}$
Temp. 60°. Mean value of $k_{uni} = 1·75 \times 10^{-2}$

From these values of k_{uni} at 50° and 60° we arrive at a value of 38,300 calories for the critical increment at p_H 5·02.

From these results it is seen that the critical increment of the heat-inactivation of trypsin is sensibly the same over the p_H range 5 to 9.

Discussion.

It may be pointed out that the critical increment of heat-inactivation of trypsin is about one-half of the critical increment of heat-denaturation of haemoglobin and about one-third of the critical increment of heat-denaturation of egg-albumin in the region of neutrality, *i.e.* the region in which the rate of denaturation is least. If heat-inactivation be connected with denaturation of the supporting or "core" protein associated with the enzyme it must follow that the protein involved is more easily denatured than is the free protein.

Again, comparing $E = 40{,}000$ calories for heat-inactivation with $E = 14{,}000$ calories for the tryptic hydrolysis of caseinogen [Moelwyn-Hughes, Pace and Lewis, 1930] and with $E = 22{,}000$ calories for the acid hydrolysis of acetylglycine [Escolme and Lewis, 1927] it is seen that inactivation is almost certainly not hydrolysis of the protein "core" of the enzyme.

It is difficult to draw any conclusions from the fact that the stability optimum of trypsin lies between p_H 6 and 7, because of the diverse results cited in the literature about the electro-chemical nature of trypsin. Thus Michaelis and Davidsohn [1911] stated that trypsin was an amphoteric body with an isoelectric point between p_H 4 and 5, while Northrop [1924] agreed that trypsin was amphoteric, but found the isoelectric point to be at p_H 10·2. Later work by Willstätter has indicated however that it is only in crude preparations that trypsin has an amphoteric character. After purification trypsin is not adsorbed by alumina suspensions having a negative charge—but is adsorbed quantitatively by kaolin suspensions which have a positive charge. From this it appears that trypsin is definitely basic in character.

It is seen that the region of optimum stability for trypsin, p_H 6–7, does not coincide with the region of optimum activity, which is p_H 7·8–8·7. In this connection, however, it should be pointed out that the rate of destruction of trypsin in a digestion mixture maintained about p_H 8·7 will not be so great as the rate of destruction found for solutions containing enzyme alone at that p_H, because in the former case the products formed during the tryptic digestion exert a considerable stabilising influence upon the enzyme.

Summary.

1. Experiments have been carried out on the heat-inactivation of trypsin which has been purified so as to be free from enterokinase and its pre-stage. The importance of this purification of an extract of pancreas is emphasised on account of the complicating effect of the "spontaneous activation" of the enzyme which otherwise occurs. It is found that the course of the heat-inactivation of trypsin purified in this way follows the unimolecular equation.

2. The effect of p_H upon the heat-inactivation of trypsin prepared in the above manner has been studied. It has been found that trypsin has an optimum stability about p_H 6·5, and that the rate of destruction increases markedly on either side of this point. The p_H-stability curve is similar in shape to that obtained for the effect of p_H upon the rate of heat-denaturation of proteins.

3. The critical increment for the heat-inactivation process has been determined (*a*) in the alkaline region, (*b*) in the acid region, (*c*) at the region of optimum stability. It is found that the critical increment is sensibly the same at all three points and is of the order of 40,000 calories per molar unit of enzyme.

The above work was carried out under the direction of Professor W. C. M. Lewis, to whom the writer is indebted for help and advice.

The writer desires to express his thanks to the Department of Scientific and Industrial Research for a maintenance grant, and also to Imperial Chemical Industries, Ltd. for a grant to the Department of Physical Chemistry of the University of Liverpool.

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LXIX. INSULIN AND INCREASE IN WEIGHT OF YOUNG ANIMALS.

By JOHN JAMES RICKARD MACLEOD, HUGH EDWARD MAGEE AND WILLIAM MIDDLETON.

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(*Received April 15th, 1930.*)

All those who have made use of rabbits for the assay of insulin have noted that most of the animals gain rapidly in weight during the first few weeks. On the other hand, according to Long and Bischoff [1930], insulin has no influence on the growth curves of rabbits. It has also been observed that depancreatised dogs treated with insulin and maintained on a diet of meat, raw pancreas and sucrose can be made to put on fat by increasing the proportion of sucrose, allowing at the same time one extra unit of insulin for every 2·5–3 g. of sugar [Macleod, 1926]. It is also well known that most diabetic patients who are allowed a liberal diet with sufficient insulin to prevent hyperglycaemia tend to put on weight rapidly. These experiences soon suggested to clinical workers that insulin might be of value in the treatment of malnutrition, particularly of children. The first observations in this direction were those of Pitfield [1923], and they have been followed by numerous others of which we will mention only those of Marriott and of Tisdall *et al.* Marriott [1924] concluded that rapid improvement in the nutrition of non-diabetic, malnourished children usually followed the intravenous injection of glucose and insulin; but this conclusion was not endorsed by Tisdall *et al.* [1925]. In view of these findings it seemed to us of interest to ascertain whether young pigs while being fed on a good fattening diet could be made to lay on fat more rapidly than ordinarily by injections of insulin. The insulin was supplied in solid form by the British Drug Houses, Ltd., and, after being dissolved in faintly acidulated water in suitable concentrations, was assayed by the rabbit method.

Experimental.

Twenty-four pigs of approximately equal weights were selected at weaning and placed on the succession of four graduated diets adopted in the Pig Department of this Institute as the most suitable for raising them for market purposes. Each diet was further supplemented by skim milk. The dry feed and skim milk, as well as water, were supplied to all animals *ad libitum*. The consumption of food per group was estimated daily, by weighing the unconsumed food. The pigs were weighed every third morning before feeding and, after three weighings, twenty animals of approximately equal weights were selected and

divided into two equal groups. The animals of Group I served as controls, and those of Group II were divided into two equal sub-groups (*a* and *b*) for injection with insulin.

The four pigs, which were not included in the above-mentioned groups, were used for studying the general effects of insulin. Two of them were deprived of food overnight and each was injected at about 10 a.m. with three units of insulin per kg. The blood-sugar, examined at hourly intervals in blood taken from the tail or ear, fell in the course of about three hours to below 0·05 % when hypoglycaemic symptoms developed, the characteristic features being rapid breathing with moderate hyperexcitability which was soon followed by muscular weakness and a state of coma with falling rectal temperature and marked cyanosis. In order to restore the animals, very large amounts of 50 % solution of glucose had to be injected subcutaneously. Although these injections were started as soon as the coma had become established, no improvement in the condition could be noted for nearly an hour. Recovery was then rapid in one of the animals, but very gradual in the other, both being, however, practically completely restored to normal by 5 p.m.

Other animals were injected with one unit of insulin per kg. body-weight, without restriction of food. The following figures show a typical effect on the percentage of blood-sugar.

	a.m.					p.m.			
Time	7·45	8.0	9.15	10.20	11.30	12.30	3.0	4.0	4.45
Blood-sugar mg. per 100 cc.	99	Insulin 1 unit per kg. Fed	69	60	73	83	94	Fed	121

In the light of these results it was decided to inject into each of the five pigs of Group II *a* 0·75, and into each of the five pigs of Group II *b* 1 unit of insulin per kg. body-weight twice daily, at 7 a.m. and 4 p.m.

The insulin was injected subcutaneously behind the ears, but no samples of blood were taken, since it was feared that the excitement and struggling which are unavoidable in collecting them might influence the results.

Results.

There was no evidence of hypoglycaemic symptoms in any of the animals throughout the period of insulin administration, which lasted 21 days in the case of Group II *b* and 59 days in that of Group II *a*. The results are shown in Tables I and II.

Table I. *Average increase in weight, actual and percentage.*

	Initial weight	Increase in weight											
	5. xi. 28	11. xi. 28		17. xi. 28		23. xi. 28		29. xi. 28		20. xii. 28		3. i. 29	
	(lb.)	(lb.)	(%)	(lb.)	(%)	(lb.)	(%)	(lb.)	(%)	(lb.)	(%)	(lb.)	(%)
Group I*	71·8	5·5	7·7	12·4	17·3	19·4	27·0	25·3	35·2	47·8	66·6	64·2	89·4
Group II *a*†	75·4	7·3	9·7	14·8	19·6	23·0	30·5	27·6	36·6	51·5	68·3	64·7	85·8
Group II *b*†	68·1	6·6	9·7	12·9	18·9	18·5	27·2	25·2	37·0‡	46·3	68·0	57·3	84·1

* Average of 10 pigs. † Average of 5 pigs. ‡ Insulin stopped.

Table II. *Average food consumption (lb.), total and "per 1 lb." gain in weight.*

Group		5. xi. 28 to 11. xi. 28		11. xi. 28 to 17. xi. 28		17. xi. 28 to 23. xi. 28		23. xi. 28 to 29. xi. 28		29. xi. 28 to 20. xii. 28		20. xii. 28 to 3. i. 29	
		R	M	R	M	R	M	R	M	R	M	R	M
I	Total	18·6	27·9	19·6	29·4	21·6	32·3	23·2	34·8	81·7	122·5	55·8	83·8
	Per 1 lb. gain	3·2	5·1	2·8	4·3	3·1	4·6	3·9	5·9	3·6	5·4	3·4	5·1
II *a*	Total	22·6	33·9	23·6	35·4	25·1	37·7	26·5	39·8	95·6	143·4	56·2	84·3
	Per 1 lb. gain	3·1	4·6	3·1	4·7	3·1	4·6	5·8	8·6	4·0	6·0	4·2	6·4
II *b*	Total	18·0	27·0	19·9	29·9	21·7	32·6	22·9*	34·4*	84·6	126·8	56·2	84·3
	Per 1 lb. gain	2·7	4·1	3·2	4·7	3·9	5·8	3·4	5·1	4·0	6·0	5·1	7·7

R Dry food. M Milk. * Insulin stopped.

It can be seen that the average weight of the animals of Group II *a* increased decidedly more rapidly than that of the controls, especially during the first three weeks of the observation, but that ultimately the controls were well ahead of the injected animals. The animals receiving the larger doses of insulin (Group II *b*) did not at first put on weight as rapidly as those of Group II *a*, but they did so more rapidly than the controls. After discontinuance of insulin in this group, the animals fell behind the controls. These results are comparable with those obtained several years ago by Miss O'Brien and one of us (J. J. R. M.) who conducted a similar series of observations on groups of full-grown rabbits. The rabbits were all fed *ad libitum* with a diet rich in readily assimilable carbohydrate and certain of them were injected twice daily with sub-convulsive doses of insulin. During the first two or three weeks, the insulin-injected animals increased in weight more rapidly than the controls, but ultimately no difference could be detected between the two groups.

Attention is to be called to the fact that the early advantage shown by the injected animals does not necessarily mean that the insulin had endowed them with a greater power of converting available carbohydrate into fat or had led to retention of water in the tissues; it may merely have stimulated the appetite so that more food was ingested. It will be seen from Table II that this was the case at least during the earlier stages of insulin injection in the pigs of Group II *a*: the total food consumption was increased but the food consumption per pound of increase in weight was unchanged. At later stages in this group for each pound of increase in weight more food was required than in the controls, indicating that the insulin was now having the effect of diminishing, rather than increasing, the assimilative powers. The results of Group II *b* show that while insulin was given there was no influence either on the total food consumption or on the assimilative power. After insulin was discontinued, the latter seemed to be decreased, since each pound increase in weight required more food than in the controls. When the pigs were ultimately sold for butchering, no difference was noted between the injected and control animals.

Although these results show conclusively that insulin does not speed up the rate of fattening of normal pigs, it does not necessarily follow that it might not have this effect when injected into poorly thriving animals. It is well known to farmers that there are often one or more pigs in a litter which do not

put on weight at the average rate, without there being any obvious pathological condition to account for the state of malnutrition.

An observation was accordingly planned in which four such underweight pigs were used, two being used as controls and two injected twice daily with one unit of insulin per kg. body-weight. The period of observation was forty-two days and the following were the results.

		Initial weight	Percentage increases in weight after 42 days
Control pigs	1	96 lb.	36·4
	2	69 lb.	23·2
Insulin pigs	1	79 lb.	44·9
	2	89·5 lb.	42·5

This indicates a decided improvement as a result of insulin, but more observations would be required before it could be concluded that injections of this hormone can with certainty convert the "runts" into normal animals.

Summary.

Two groups of 5 healthy pigs were given insulin daily in subcutaneous doses of 1·5 and 2 units per kg. body-weight and their weights compared with those of 10 animals fed on the same diet but receiving no insulin.

Insulin had only a very slight accelerating effect on the rate of increase in weight, which was evidently due to a correspondingly greater consumption of food and not to any metabolism-sparing action.

Other experiments suggested that insulin had a more marked effect on marasmic pigs.

We desire to express our thanks to Mr D. W. Auchinachie, and Mr Wm Thomson for technical assistance.

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LXX. THE CHARACTERISTIC ABSORPTION OF ULTRA-VIOLET RADIATION BY CERTAIN PURINES.

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(*Received April 18th, 1930.*)

The measurement of the absorption of ultra-violet radiations by biological substances, as effected with the spectrophotometer, has been used with some success in recent years. Using this method Smith [1928] measured the ultra-violet absorption of uric acid and pointed out the close correspondence of the absorption of an ultra-filtrate of human plasma with that of a simple solution of uric acid.

In some unpublished work, the present writer has found that ultra-filtrates of certain other body fluids and tissue extracts have markedly different absorptions from that of plasma. The substances most likely to cause this absorption would seem to be purines.

It was therefore decided to establish the characteristic absorption of these substances and so obtain a basis for future investigation, both quantitative and qualitative, of body fluids. In this paper the results of this investigation are reported.

Historical.

Soret [1883] and Hartley [1905] investigated the absorption of purines in the ultra-violet region. Later Dhéré [1909] examined purines and pyrimidines and showed that they all exhibited selective absorption. He also demonstrated that uric acid shows one absorption band in alkaline solution and two bands in acid solution. These observations were not quantitative, owing to the limitations of the methods employed.

Smith [1928], using a Hilger spectrophotometer with rotating sector, was able to measure the absorption of uric acid quantitatively, and confirmed Dhéré's observations in acid and alkaline solutions. Recently Marchlewski and Wierznchowska [1929] have investigated adenine, guanine and uric acid in the same way; they failed however to observe the effect of hydrogen ion concentration on the ultra-violet absorption spectra.

Methods.

The absorption spectra were measured with a Hilger quarter-plate spectrophotometer with rotating sector. The source of radiation was a condensed spark between tungsten-steel electrodes. A Hilger micrometer cell was used for determining whether Beer's law was followed. The thickness of the cell could be varied from 0 to 5 mm., the micrometer being graduated in 0·01 mm. and reading to 0·01 mm. with 5 % error.

Ilford process plates were used except where investigation of the spectrum of shorter wave-lengths than 2150 Å. was desired, in which case Schumann plates (Hilger) were employed.

Experimental.

Each purine was examined with the object of showing:

(1) whether Beer's law was followed;

(2) the effect of hydrogen ion concentration on the absorption spectra;

(3) the effect of chemical combination of the purine with a molecule which has no appreciable absorption at the same wave-length.

The purines were examined in as pure a state as possible. Guanine and adenine and their nucleotides were isolated from yeast nucleic acid by the method of Jones [1914]. Hypoxanthine was obtained from meat extract. The adenosine was a commercial product and two commercial samples of uric acid were investigated. The nitrogen content was taken as the standard of purity and recrystallisation repeated until the estimated content agreed within 1 % of the theoretical value.

(1) *Beer's law*[1].

The solvent in all cases was water.

The thickness of the cell was varied so that in the formula

$$E = \frac{1}{cd} \log \frac{I'}{I}$$

$c \times d$ was constant. The sector used was one of the short focus type which will not hold a cell of more than 1 cm. length. The experimental error of the spectrophotometric method is least at an ϵ value on the rotating sector of

[1] Beer's law states that the absorptive power of molecules is independent of their concentration. This can be expressed graphically by

$$\log \frac{I}{I'} \times \frac{1}{cd} = \text{constant},$$

where I is the intensity of the incident beam of monochromatic light and I' the intensity of the same light after transmission through a layer of absorbing solution of thickness d and concentration c.

The ratio $\log \frac{I}{I'}$ is expressed as ϵ (the extinction coefficient) and is given by the reading on the sector. E (the specific extinction coefficient) has been taken in this paper as being the constant in the above expression. Molecular E is this constant when $d = 1$ cm. and c is the molecular weight of the substance in grams in one litre and is of importance only for purposes of comparison, having no practical meaning.

1·0 to 1·1. This results from two opposing sources of error. The lower the ϵ value on the rotating sector the higher the percentage error, due to the error of the spectrophotometer being constant over all ranges [von Halban and Eisenbrand, 1927]. On the other hand, the higher the ϵ value on the sector the more difficult is it to set the sector accurately owing to the scale divisions being logarithmically spaced and very close at high ϵ values. A mean is struck at $\epsilon = 1{\cdot}0$–$1{\cdot}1$ where the mechanical error is low and the accurate setting of the sector relatively easy. From these considerations the weakest solution was made so that with a thickness of 1 cm. ϵ had a value of 1·1.

Five dilutions were examined ranging from the weak to a saturated solution. Owing to the slight solubility of the purines in water the range is not large, the saturated solution having about 25–40 times the concentration of the weakest solution examined.

All the purines examined follow Beer's law within the limits investigated.

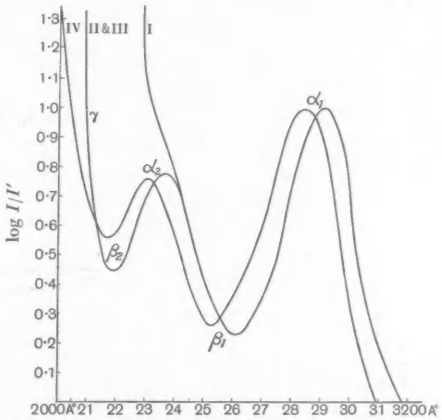

Fig. 1. Uric acid. Effect of hydrogen ion concentration on absorption curve.
I $p_H = 9{\cdot}1$. II $p_H = 7{\cdot}8$ III $p_H = 6{\cdot}8$ IV $p_H = 2{\cdot}0$

(2) *Hydrogen ion concentration.*

Owing to their general absorption at the shorter wave-lengths, buffer solutions were found unsuitable for obtaining solutions of given hydrogen ion concentration. Solutions of purines in suitable strength were made up in alkali (NaOH) and titrated with acid (HCl) to an approximate p_H. This was subsequently determined by the glass electrode method [Kerridge, 1925]. The results are shown in Tables I and II. Where the effect of hydrogen ion concentration is small, determinations at only two p_H values are given.

Absorption curves of uric acid and guanine showing the effect of hydrogen ion concentration are given in Figs. 1 and 2.

Table I.

	p_H	Molecular E: α apex	Molecular E: β base	Apex/base ratio α/β	Wave-length (Å.): α	β	γ
Adenine	3·0	$1{\cdot}4\times10^5$	$0{\cdot}26\times10^5$	5·4	2630	2300	2110
	10·0	$1{\cdot}4\times10^5$	$0{\cdot}26\times10^5$	5·4	2660	2380	2230
Adenine nucleotide	4·0	$1{\cdot}3\times10^5$	$0{\cdot}24\times10^5$	5·5	2620	2290	2110
Adenosine	8·0	$1{\cdot}3\times10^5$	$0{\cdot}24\times10^5$	5·5	2640	2340	2200
Hypoxanthine	3·0	$0{\cdot}9\times10^5$	$0{\cdot}15\times10^5$	5·6	2450	2210	2070
	7·0	$0{\cdot}9\times10^5$	$0{\cdot}15\times10^5$	5·6	2490	2220	2080
	11·0	$0{\cdot}9\times10^5$	$0{\cdot}15\times10^5$	5·6	2590	2300	2170
Caffeine	3·0	$0{\cdot}8\times10^5$	$0{\cdot}14\times10^5$	5·6	2740	2440	2180
	10·0	$0{\cdot}8\times10^5$	$0{\cdot}14\times10^5$	5·6	2710	2430	2190
Theobromine	3·0	$0{\cdot}9\times10^5$	$0{\cdot}3\times10^5$	3·0	2720	2410	2190
	10·0	$0{\cdot}9\times10^5$	$0{\cdot}3\times10^5$	3·0	2740	2480	2210
Ergothioneine	—	$1{\cdot}1\times10^5$	—	—	2570	—	—

Table II.

	p_H	Molecular E: α_1	β_1	α_2	β_2	Wave-length (Å.): α_1	β_1	α_2	β_2	γ
Uric acid	9·1	$1{\cdot}0\times10^5$	$0{\cdot}25\times10^5$	—	—	2920	2610	—	—	2315
	7·8	$1{\cdot}0\times10^5$	$0{\cdot}25\times10^5$	$0{\cdot}76\times10^5$	$0{\cdot}45\times10^5$	2920	2610	2360	2190	2090
	6·7	$1{\cdot}0\times10^5$	$0{\cdot}25\times10^5$	$0{\cdot}76\times10^5$	$0{\cdot}45\times10^5$	2920	2610	2360	2190	2090
	2·0	$1{\cdot}0\times10^5$	$0{\cdot}25\times10^5$	$0{\cdot}76\times10^5$	$0{\cdot}55\times10^5$	2840	2530	2300	2170	2040
Guanine	9·0	$0{\cdot}63\times10^5$	—	—	$0{\cdot}43\times10^5$	2760	—	—	2300	2240
	8·8	$0{\cdot}63\times10^5$	—	—	$0{\cdot}43\times10^5$	2760	—	—	2300	2200
	6·8	$0{\cdot}63\times10^5$	$0{\cdot}52\times10^5$	$0{\cdot}64\times10^5$	$0{\cdot}43\times10^5$	2760	2620	2500	2300	2180
	2·4	$0{\cdot}63\times10^5$	$0{\cdot}52\times10^5$	$0{\cdot}82\times10^5$	$0{\cdot}33\times10^5$	2760	2620	2500	2230	2120
	0·87	—	—	$1{\cdot}09\times10^5$	$0{\cdot}32\times10^5$	—	—	2480	2230	2090
Guanine nucleotide	1·0	—	—	$1{\cdot}05\times10^5$	$0{\cdot}26\times10^5$	—	—	2500	2230	2090

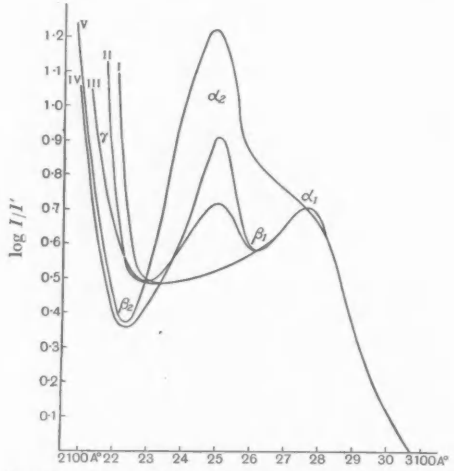

Fig. 2. Guanine. Effect of hydrogen ion concentration on absorption curve.

I $p_H=9{\cdot}0$ II $p_H=8{\cdot}8$ III $p_H=6{\cdot}8$ IV $p_H=2{\cdot}4$ V $p_H=0{\cdot}87$

Discussion of results.

It will be seen that purines can be divided into two groups: (1) those which have only one absorption band (Table I), and (2) those which have two bands in acid solution (Table II). The absorption of the first group is little influenced by increase in hydrogen ion concentration, the only effect being a slight shift of the absorption towards the shorter wave-lengths. The average shift is about 30 Å. with a change from p_H 10·0 to p_H 3·0. The exception is hypoxanthine, which shows a shift of 140 Å. over the same range of p_H. The extinction coefficient is unaffected in all cases by change of hydrogen ion concentration, which is in disagreement with Smith [1928], who found a slightly higher extinction coefficient for uric acid in alkaline than in acid solution.

The second group comprises uric acid and guanine. In the case of uric acid (Fig. 1) change of the absorption takes place in two stages: (i) between p_H 9·1–7·8 (curves I and II) and (ii) between p_H 6·8–2·0 (curves III and IV). No change occurs between p_H 7·8–6·8 (curves II and III). Two dissociation constants for uric acid [His and Paul, 1900] are given as 2×10^{-6} and $2{\cdot}0 \times 10^{-9}$ which correspond to p_H values of 5·7 and 8·7 respectively. It seems probable, therefore, that in the case of uric acid the absorption changes correspond to changes between acidic and basic forms of the molecule.

In the case of guanine the change in the absorption seems to be a gradual one throughout the range of p_H. One dissociation constant is given for guanine by Wood [1903] as $8{\cdot}4 \times 10^{-12}$, which corresponds to a p_H of 11·0. This is outside the range shown in Fig. 2, but a solution of guanine at that p_H shows the same absorption curve as I in Fig. 2. A titration curve of guanine showed no evidence of a second dissociation constant. From its constitution as a 2-amino-6-hydroxypurine one would expect guanine to have two dissociation constants. If, as has been suggested in the case of uric acid, absorption changes result from intramolecular rearrangements in the dissociation of guanine, it can be seen that the dissociation into the more basic form is spread over a wide range. This is probably the reason for the difficulty in determining the second dissociation constant.

The results show the importance of determining the hydrogen ion concentration in any spectrophotometric method of estimating or identifying purines.

It will be seen that chemical combinations have no effect on the absorption of the purines as judged by the absorption spectra of their nucleosides or nucleotides. The absorption of these substances is identical with that of the corresponding purines under the same conditions.

In general it may be said that the spectrophotometric method offers a fresh mode of attacking the problems of purine metabolism. It must be emphasised, however, that it can only be an adjunct to the usual biochemical methods of analysis and is useless by itself for investigating tissue fluids.

Even if the substances causing selective absorption in these fluids are purines, it can be seen from the tables that the absorption bands of the different members occur so close to one another that it would be impossible to identify and estimate two such occurring in roughly equivalent amounts. On the other hand, as a rapid method of estimating separate members of the purines the method is of great value, and the fact that nucleosides and nucleotides are estimated in terms of the contained purine might be made use of in estimating proportions of free and combined purine. In the case of uric acid, which Benedict [1923] states occurs in ox-blood combined as a riboside, the spectrophotometric method has shown, in preliminary experiments by the writer, that, by a simple method of difference, an estimate of the amount of combined uric acid can be obtained. The chemical methods only estimate free uric acid and the spectrophotometric method total uric acid, free and combined.

The chemical methods are few and of doubtful reliability. The results for uric acid in normal human blood are probably of fair accuracy, but this does not apply to animal blood [Bulmer, Hunter and Eagles, 1925]. Buell and Perkins [1928] have developed a nephelometric method for determination of adenine nucleotide in blood but, apart from these, no methods for estimating purines in small amounts of blood are available. It therefore seems reasonable to hope that the spectrophotometric method, if used with proper precautions, may be of value in investigations of purine metabolism.

It was thought of interest to include observations on ergothioneine. This compound, thiolhistidinebetaine, exists in blood and interferes with colorimetric determinations of uric acid. It was isolated from pig's blood by Hunter and Eagles [1925]. It shows selective absorption very similar to that of purines and the molecular extinction coefficient is of the same order. The interest of its absorption spectrum lies, however, in the fact that it is completely transparent to rays of short wave-length as far as 1900 Å.

SUMMARY.

1. The ultra-violet absorption spectra of the following purines has been determined: uric acid, guanine, adenine, hypoxanthine, caffeine and theobromine.

2. Beer's law is followed by all.

3. The effect of hydrogen ion concentration on the absorption spectra has been measured and its importance emphasised.

4. Combination of a purine in the form of a nucleoside or nucleotide does not alter the absorption.

5. The ultra-violet absorption spectrum of ergothioneine has also been determined.

The writer wishes to express his indebtedness to Dr J. R. Marrack for the hydrogen ion determinations, to Dr H. D. Kay for the adenine and guanine

nucleotides, to Dr Eagles for the sample of ergothioneine and to Mr Campbell Smith for many valuable suggestions.

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LXXI. THE ISOLATION OF HISTAMINE FROM THE HEART.

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In connection with some problems on the physiology of the heart under investigation in this laboratory, the question arose as to whether the readily extractible depressor substance was the base histamine.

It is well known that simple aqueous or alcoholic extracts of almost any tissue cause a fall in blood-pressure on injection into the circulation of the cat. The heart is not an exception. In fact, it yields an extract with powerful depressor activity.

The marked similarity in the physiological behaviour of tissue extracts and of solutions of histamine gave rise to the view that histamine was present in these extracts, a view which has been confirmed recently by the isolation of pure histamine, in the form of its dipicrate, from extracts of liver and lung [Best, Dale, Dudley and Thorpe, 1927], of muscle [Thorpe, 1928], and of spleen [Dale and Dudley, 1929]. It therefore seemed reasonable to expect that the depressor substance of heart extracts would be histamine. This expectation has, in fact, been realised.

Apart from giving additional confirmation to the view that extracts of most tissues contain histamine, proof of the presence of histamine in heart extracts is of significance in connection with recent work on the so-called "heart hormone." Many workers have observed that heart extracts have a stimulating action on the frog's and rabbit's heart but there is much disagreement as to the nature of the substance responsible for these effects. Some workers [Demoor, 1924; Haberlandt, 1927] are of the opinion that the substance is formed in certain regions of the heart and is a specific heart hormone. Others, however [Rigler and Tieman, 1927; Rigler, 1929; Oppenheimer, 1929], deny the specificity of this substance, and have obtained extracts producing similar effects not only from any region of the heart but also from a variety of other tissues. Further, they have produced similar effects with pure histamine alone. Rigler, in fact, on physiological grounds puts forward the view that the substance is actually histamine. A full discussion of the work in this field is beyond the scope of this paper. The main features are briefly but effectively summarised by Oppenheimer [1929], who gives references to the more important papers.

The experiments described in the present communication prove that histamine is present in extracts of ox ventricles. Further, these extracts appear to contain relatively large quantities of histamine. In view of the presence of this large amount of histamine in heart extracts, it seems probable that the cardiac stimulating effect of heart extract may be due wholly or in part to histamine, and it is clear that unless histamine is removed from such extracts no conclusion can be drawn with regard to the presence of a specific heart hormone. In the absence of evidence for a specific heart hormone, chemical proof of the presence of histamine in heart extract tends to support Rigler's view.

The vasodilator activity of different tissue extracts varies considerably [Thorpe, 1928]. Lung extracts are particularly rich in histamine compared to other tissue extracts. Heart extract was found to have activity of a high order, being second only to lung extract.

Extracts prepared from four ox hearts (1·3, 1·5, 1·2, 1·7 kg.) gave values equivalent to 9·4, 18, 9·6, 8·1 mg. histamine per kg. of fresh tissue respectively when estimated by comparison with pure histamine on the blood-pressure of the cat under chloralose. The action was all of a histamine-like type and there was neither chemical nor physiological evidence to suggest the presence of a choline-like substance.

The above values apply to the ventricles of the heart. In two cases the auricles were worked up giving the interesting result that the activity, weight for weight of fresh tissue, was approximately double that of the ventricles of the heart in question. Auricle extracts therefore have nearly the same order of activity as lung extracts (40 mg. per kg.).

The technique employed for the isolation of histamine from heart extract was similar to that employed previously, alcohol being used for the extraction. In the final stages difficulties arose similar to those encountered in dealing with muscle extracts [Thorpe, 1928]. Heart muscle contains considerable quantities of creatine which during the silver fractionation is converted to creatinine and methylguanidine. The presence of these substances made the isolation of histamine picrate very difficult. The total amount of bases in the histidine and arginine fractions was small and it was found that the alcohol extraction of these could be omitted.

With regard to the freshness of the material, the hearts were obtained in the laboratory within half an hour of the animals' death at the slaughter house and the same precautions were taken to ensure sterility as in previous work.

Experimental.

The ventricles from a fresh ox heart (1·5 kg.) were minced and extracted for 24 hours in 3·5 l. 96 % alcohol, the extract was filtered off in a press and the meat re-extracted with a further 3 l. 60 % alcohol. The combined extracts, after addition of sulphuric acid (0·36 cc. 50 % per l.) which produced a slight precipitate, were filtered and concentrated *in vacuo* below 30° to about 250 cc.

After removal of fat by shaking with ether, the fat-free aqueous layer was concentrated to 160 cc. After removal of 2·5 g. creatine which separated on standing, the liquid was neutralised with NaOH and treated with basic lead acetate until no further precipitate was produced. The filtrate and washings from this precipitate, after removal of lead as sulphide, were concentrated *in vacuo* to 100 cc. when a further 1·65 g. creatine separated.

After removal of creatine, 50 % sulphuric acid was added to produce a concentration of 5 %. Phosphotungstic acid (25 % in 5 % sulphuric acid) was then added until precipitation was complete, 400 cc. being required. The precipitate was decomposed in the cold with baryta in the usual way, excess of baryta being removed as sulphate. The solution of bases was concentrated to 100 cc. and fractionated by the Kossel-Kutscher method. 15 cc. 40 % silver nitrate were added and the resulting precipitate ("purine" fraction) removed. Hot saturated baryta was then added cautiously until the white precipitate was just tinged with brown. The precipitate ("histidine" fraction) was filtered off. Saturation of the filtrate with baryta precipitated the "arginine" fraction. The filtrate from this precipitate forms the "lysine" fraction.

The purine and lysine fractions had no appreciable activity. Silver was removed from the histidine and arginine fractions by suspending in sufficient dilute sulphuric acid to render the liquid just acid to Congo red and treating with hydrogen sulphide. After removal of silver sulphide excess of sulphuric acid was removed by baryta and the solution of the bases from each fraction evaporated to dryness *in vacuo*.

A biological assay of the solutions before evaporation had indicated that the depressor activity of the histidine and arginine fractions was equivalent to 3·2 and 4·8 mg. histamine respectively.

The histidine fraction was treated with picric acid which immediately gave a crystalline precipitate. After a long fractionation of the picrate 0·25 g. potassium creatinine picrate and 0·1 g. creatinine picrate were separated in pure condition. The residues showed a powerful histamine-like activity but it was not found possible to isolate histamine picrate in a pure state from the creatinine picrate still remaining.

The arginine fraction contained relatively little material and was also converted to picrate. This picrate (180 mg.) was subjected to a long fractional crystallisation similar to that employed when dealing with muscle extract. The mixture consisted mainly of the picrates of methylguanidine and histamine and finally after resorting to mechanical separation of the crystals a total of 5 mg. pure histamine dipicrate was obtained. At the same time 50 mg. methylguanidine picrate were obtained.

A solution of 3·2 mg. of this histamine dipicrate in 100 cc. water had the same depressor effect on the cat's blood-pressure as a similar solution prepared from a known sample of pure histamine dipicrate. The Pauly reactions were quantitatively equal. The picrate from heart, pure histamine dipicrate and a mixture of the two all melted together at 240° with decomposition.

The yield of histamine from heart.

Throughout the purification samples were taken at the end of each stage for assaying on the cat's blood-pressure in comparison with pure histamine. The values obtained are given in Table I.

Table I.

Stage	Activity in mg. histamine
Crude extract	27
After basic lead acetate	22
Phosphotungstate	14
Purine fraction	— 0·3
Histidine fraction	— 3·2
Arginine fraction	4·8
Lysine fraction	— 0·0
Histamine isolated as pure picrate	1·0

Although rather better than that from muscle, the yield of pure histamine from heart extract is poor. But if the yields are considered at the end of each stage in the purification the losses in the earlier stages are reasonable, and comparable with those obtained in experiments with other tissues, only the loss in the silver fractionations being rather larger than usual (41 % instead of about 25 %). The yield from the picrate stage is certainly low, but it must be remembered that the experiment was performed on a very small scale. Consequently, since sufficient volume of liquid had to be left for manipulation, relatively less creatine could be removed by crystallisation in the early stages than in large-scale experiments such as those with muscle. Thus a relatively greater interference by creatinine and methylguanidine is to be expected in the picrate stage.

The physiological assays show a continuous fall in activity throughout the purification, a fall which would be expected if histamine was adsorbed by the relatively large precipitates removed during the manipulation. The losses are of the same order as in previous experiments with other tissues, and it would appear that the losses in activity are due to the removal of histamine and not to the removal of another depressor substance. This is supported by the fact that the biological assays gave no hint of the presence of any substance other than one closely resembling histamine, so that it seems justifiable to conclude that the depressor substance of heart extract prepared as described in this paper is the base histamine.

SUMMARY.

(1) Extracts of ox ventricles show a powerful depressor activity corresponding to 8 to 18 mg. histamine per kg. assayed biologically.

(2) Pure histamine, in the form of its dipicrate, has been isolated from such an extract and it is suggested that this substance is responsible for the depressor activity.

(3) The significance of these findings in relation to recent work on the so-called heart hormone is discussed.

I wish to express my gratitude to Prof. I. de Burgh Daly for carrying out a number of the physiological assays required during these experiments.

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LXXII. THE ABSORPTION AND EXCRETION OF CALCIUM AND PHOSPHORUS BY RATS RECEIVING EXCESSIVE DOSES OF IRRADIATED ERGOSTEROL.

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Report to the Medical Research Council.

(*Received April 26th, 1930.*)

It has been shown by Kreitmair and Moll [1928], Harris and Moore [1928, 1929], and Kreitmair and Hintzelmann [1928], and has since been confirmed by many other workers, that the administration of excessive doses of irradiated ergosterol to animals results in the production of calcium deposits, notably in the kidney and aorta, and in a tendency to hypercalcaemia and hyperphosphataemia. There have been two theories put forward to account for the source of this extra calcium in the blood and in certain tissues. One, first suggested by Harris and Moore, postulates an increased absorption from the gut together with an increased retention. Smith and Elvove [1929] suggested diminished excretion of calcium, though they did not indicate whether this decreased elimination is by way of the gut or the kidney, or both. The second theory is that the source of hypercalcaemia and depositions is the calcium of the bone substance. This has been suggested by Hess, Weinstock and Rivkin [1929–30], Baumgartner, King and Page [1929], Light, Miller and Frey [1929] and others.

Hoyle and Buckland [1929] made a study of the urinary phosphorus in hypervitaminosis D, but did not estimate the calcium. Light, Miller and Frey [1929] have given figures for calcium and phosphorus balances, but owing to the fact that no figures for food intake, urine or faeces were published it is not possible to judge what processes were at work to produce the results obtained by them. An investigation of the urinary calcium in some detail (as was done by Hoyle and Buckland for the phosphorus) followed by total balance experiments was therefore undertaken, and is described below. Just as the work was completed a paper by Brown and Shohl [1930] was published, in which they showed a definitely decreased retention of calcium after large doses of irradiated ergosterol, and a shift in the excreted calcium from the faeces to the urine. It was felt that independent confirmation of their results was probably worth publication, especially as some new points have emerged.

I. Urinary excretion in hypervitaminosis D.

Methods.

Calcium. Shohl and Pedley's [1922] method was used, with slight modifications. As rats' urine seems to have given trouble to other workers (see Hoyle and Buckland for a discussion of the difficulties) the final routine adopted is described in full. Urine and washings from the receiving flask, approximating to 100 cc., is treated with 10 cc. concentrated sulphuric acid and 4 g. ammonium persulphate, as described by Shohl and Pedley. As the urine is heated a large bulky precipitate separates. This precipitate also occurs, though to a lesser degree, in human urine, whereas there is no trace of it in rabbit urine. By continuing the heating for 2 or 3 hours (instead of 1 hour as recommended by Shohl and Pedley), adding more ammonium persulphate from time to time whenever the urine begins to darken in colour, the precipitate becomes much reduced in amount, but does not entirely disappear. If the calcium is precipitated in its presence the final titration with permanganate gives results which are much too high. It must, therefore, be filtered off and washed twice with small quantities of hot hydrochloric acid, the washings being added to the treated urine. This washed precipitate when dissolved in a small quantity of alkali gives no reactions when tested for urates, calcium or inorganic phosphate. The precipitation of calcium can now be carried out as described in the original method. If small amounts of calcium are present, as in normal rats' urine, it is advisable to allow 24 hours for complete precipitation.

Phosphorus. The filtrate and washings from the calcium precipitate were made up to 250 cc. and used for the colorimetric estimation of phosphate, after further suitable dilution. The amount of oxalic acid present in the final dilution is too small to interfere with the colour development. The Bell-Doisy reagents [1920] were used instead of those of Briggs.

Experimental.

Eight rats, four males and four females, were kept in Hopkins's metabolism cages, two rats in each cage. The urine from each cage was kept and analysed separately, three or four days' output being mixed together. The weights of the animals were between 100 and 168 g. The diet was identical with that used by Harris and Moore [1928]. Full synthetic diet was given for 14 days before administration of the irradiated ergosterol, which was then fed at 0·1 % level. Urine was collected for 1 week only during the preliminary period. It was found essential that the animals should be kept in the metabolism cages for a few days before collecting urine, as, until they became accustomed to the change of cage, metabolism was apt to be appreciably disturbed. After 20 days of the irradiated ergosterol administration the animals were put back on to normal synthetic diet for the "curative" period. During the course of the experiment the females showed greater loss of weight

than the males; two of them died at the beginning of the "curative" period. Post-mortem examination revealed the usual lesions associated with hypervitaminosis D in an advanced form. Four of the rats had some diarrhoea during the intake of irradiated ergosterol; consequently one or two samples of urine had to be discarded as they were badly contaminated with faeces.

The results of this experiment are given in Table I.

Table I. *Urinary excretion of calcium and phosphorus (mg. per rat per day).*

Group I					Group II				
	Females		Males			Females		Males	
No. of days	Ca	P	Ca	P	No. of days	Ca	P	Ca	P
				Preliminary period.					
4	1·3	25·7	0·8	26·5	4	0·1	21·8	0·1	26·7
4	0·3	23·9	0·7	24·3	3	0·1	30·0	0·1	28·9
				Irradiated ergosterol period.					
3	5·9	25·4	5·9	27·3	4	4·5	17·2	5·3	18·5
3	5·3	17·9	7·9	24·6	4	8·3	24·5	8·9	22·6
4	2·3	15·1	3·2	10·5	4	9·9	23·5	10·1	23·8
					4	8·8	23·7	8·9	28·3
					4	8·1	23·5	8·4	25·9
				"Curative" period.					
4	—	—	2·3	17·8	3	7·3	22·4	7·0	19·1
3	—	—	1·2	30·9	3	4·3	20·4	3·8	24·2
3	—	—	1·4	28·2	2	2·2	16·8	2·0	21·3

Discussion.

These two groups of rats agreed in showing the great increase in urinary calcium as a result of the massive doses of irradiated ergosterol; this was more marked in the second group. This group did not show the sudden decrease in the calcium excretion towards the end of the irradiated ergosterol intake. The calcium in the urine of both males and females of the second group showed a rise to a maximum occurring at about the tenth day, and then followed a slow and steady decline. The concentration of calcium rose steadily throughout the period of irradiated ergosterol administration in the case of the females, whereas in the case of the males the concentration was roughly parallel to the total daily amount of calcium excreted. There was a sharp drop in the calcium concentration about 3 days after the initiation of the "curative" period (Figs. 1 and 2). The diuresis found by Hoyle and Buckland [1929] was not shown by any of these rats. The worse physical condition of the animals in the first group may explain the drop in the urinary calcium towards the end of the irradiated ergosterol period; kidney function was probably failing rapidly, as the phosphate showed the same decline and the volume of urine passed was very small. There was no increase in phosphorus excretion produced by the hypervitaminosis. This is in agreement with Hoyle and Buckland.

It is thus evident that decreased excretion of calcium by the kidney is not responsible for the hypercalcaemia or for the calcification of tissues.

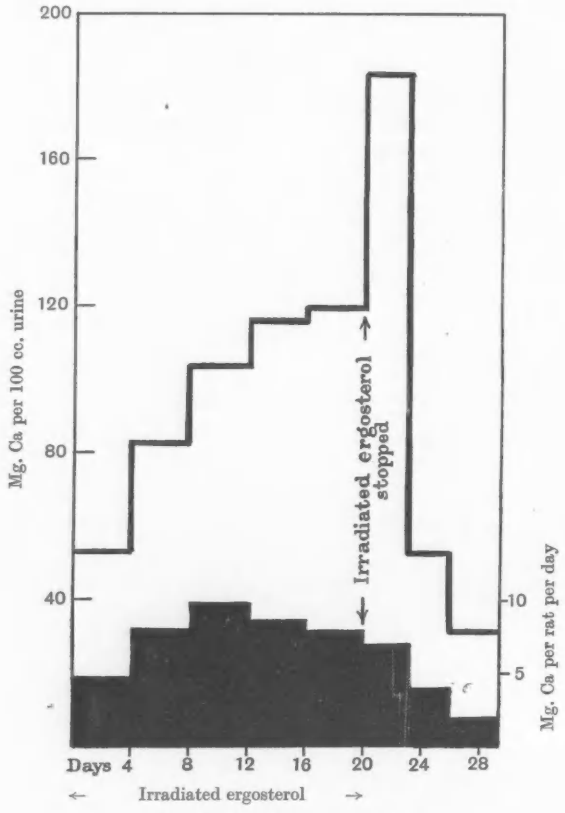

Fig. 1. ♀ Daily excretion of Ca.

II. Total balance experiments.

Methods.

The technique for the urine analyses was as described above. Calcium in the faeces was determined by a slight modification of McCrudden's method [1910] rendered necessary by the fact that there was sometimes insufficient faeces to admit of a separate phosphorus estimation. The following technique was therefore adopted in all cases to ensure uniformity. After ashing, the ash was dissolved in 5 cc. concentrated sulphuric acid, and heated with about 10 drops of perhydrol[1]. This gave a clear solution, and converted any pyrophosphate present as the result of the ashing process into orthophosphate,

[1] This method of treating the ash was suggested to me by Mr E. G. Holmes, to whom my thanks are due.

which could be estimated colorimetrically. The dissolved ash in the sulphuric acid was diluted to 100 cc. with distilled water, and the calcium estimated as described by McCrudden. The filtrate and washings from the calcium precipitate were made up to 250 cc. and the phosphorus determined as in the case of urine.

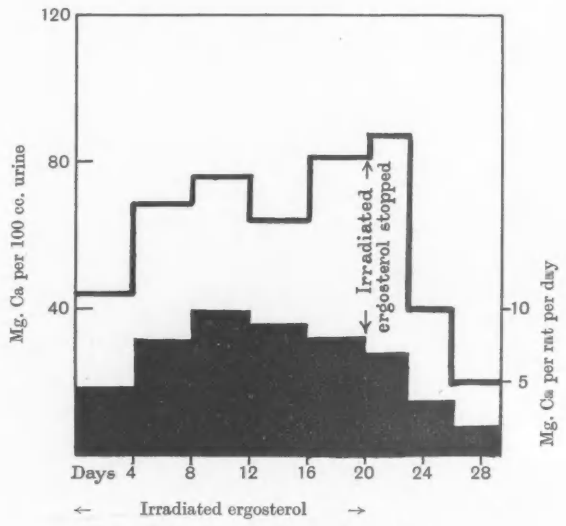

Fig. 2. ♂ Daily excretion of Ca.

Experimental.

Six albino male rats of approximately the same age were used. Each animal was kept in a separate metabolism cage, and faeces and urine were collected separately in 3 or 4 day lots. The daily amount of food fed was weighed, the dry weight of an equivalent amount determined, and any uneaten residues collected, dried and weighed. In this way the exact quantity eaten could be obtained by subtraction. Distilled water was given for drinking. The experiment as before was divided into three parts, (1) preliminary period, (2) irradiated ergosterol period and (3) "curative" period. The irradiated ergosterol was given at 0·05 % level, this avoids the complication of soft faeces or diarrhoea, yet is sufficient to cause loss of weight and pathological lesions. The weights of the animals (g.) are given below.

No. of rat	F5	F6	F7	F8	F21	F22
Beginning of irradiated ergosterol intake	135	139	159	155	160	157
End of irradiated ergosterol intake	116	123	141	142	144	143
End of "curative" period	144	150	167	164	155	160

Table II.

No. of rat	Ca intake Periods I	II	III	Faecal Ca Periods I	II	III	Urinary Ca Periods I	II	III	Total Ca excretion Periods I	II	III	Ca retention Periods I	II	III
F5	24·2	18·0	30·1	7·2	6·9	19·8	1·5	6·6	4·9	8·7	13·5	24·7	15·5	4·5	5·4
F6	29·4	25·9	38·4	3·8	1·4	6·2	1·6	9·4	5·8	5·4	10·8	12·0	24·0	15·1	26·4
F7	33·4	21·9	36·4	9·7	6·5	21·2	2·3	7·4	3·3	12·0	13·9	24·5	21·4	8·0	11·9
F8	26·8	26·7	34·6	8·1	6·8	11·4	1·3	5·9	4·9	9·4	12·7	16·3	17·4	14·0	18·3
F21	28·6	20·2	35·2	16·7	10·1	23·9	0·1	5·3	3·6	16·8	15·4	27·5	11·8	4·8	7·7
F22	28·1	18·9	32·1	16·9	5·9	19·9	0·2	6·4	4·4	17·1	12·3	24·3	11·0	6·6	7·8

Figures represent mg. per rat per day.
Period I. Preliminary.
" II. Irradiated ergosterol intake.
" III. "Curative" period.

Table III.

No. of rat	P intake Periods I	II	III	Faecal P Periods I	II	III	Urinary P Periods I	II	III	Total P excretion Periods I	II	III	P retention Periods I	II	III
F5	49·3	36·6	64·7	7·2	7·1	16·2	39·8	31·0	36·6	47·0	38·1	52·8	2·3	−1·4	11·9
F6	59·6	52·6	78·0	5·6	3·8	4·8	36·0	36·2	39·7	41·6	40·0	44·5	18·0	12·6	33·5
F7	67·9	44·7	74·0	10·4	8·5	18·4	40·4	35·2	36·4	50·8	43·7	54·8	17·1	1·0	19·2
F8	54·4	54·2	70·1	9·4	8·9	16·0	29·2	37·4	40·7	38·6	46·3	56·7	15·8	7·9	13·4
F21	58·1	41·0	71·2	11·0	10·1	23·6	28·6	38·2	39·7	39·6	48·3	63·3	18·5	−7·3	7·9
F22	57·0	37·7	65·4	12·4	5·2	19·2	29·2	28·1	36·9	41·6	33·3	56·1	15·4	4·4	9·3

Figures represent mg. per rat per day.
Period I. Preliminary.
" II. Irradiated ergosterol intake.
" III. "Curative" period.

The intake, output, and retention of calcium and phosphorus are given in Tables II and III. The following points are shown by an examination of the figures. (1) The urinary excretion was much the same as in the case of the rats receiving the higher dose of irradiated ergosterol. (2) The irradiated ergosterol period was characterised by a decrease (sometimes very marked) of the faecal calcium. Only two cases (F6, F22) showed a marked change in the phosphorus. (3) The urinary calcium did not return to normal in the "curative" period during the time of the experiment (9 days generally). Evidently the recovery from the effects of the hypervitaminosis is only gradual, as one would expect. (4) The faecal calcium in the recovery period tended to be very high—much higher than it was in the normal preliminary period, so that the total excretion of calcium was the highest of all in the "curative" period. The calcium intake was also highest in this period, yet even so, in two cases (F5, F7) the extra intake compared with that of the preliminary period was 6 and 3 mg. per day, respectively, while the extra output in the faeces was 12 and 11 mg. per day respectively. (5) The faecal phosphorus was definitely increased in the "curative" period in all cases except one (F6). (6) The balance of calcium was never negative when averaged for the whole period of irradiated ergosterol administration (see however later remarks on this point). In every case the balance was materially reduced by the

hypervitaminosis, and in three cases was not restored to normal during the observed period of recovery. (7) The phosphorus balance was also considerably reduced by the irradiated ergosterol.

It was not felt that the average balances for the whole of the irradiated ergosterol and "curative" periods respectively gave an adequate picture of what was taking place. These balances have therefore been further analysed in Tables IV and V. Only two 3-day collections of excreta were made from rats F5 and F7, as they were in such bad condition at the end of this short time that it was not advisable to push the experiment further. Rats F6 and F8 were observed for only 6 days instead of 9 in the recovery period. In these tables also are given the so-called "percentages of absorption" of the two elements. These have been calculated tentatively as follows: the faecal excretion was subtracted from the intake and the remainder counted as the amount absorbed, and expressed as a percentage of the intake. This is not strictly accurate, because the faecal calcium consists both of calcium which has never been absorbed and of some which has been absorbed and passed back into the gut. There is no way at present of distinguishing these two fractions, so that the above method of calculating the amount absorbed is the only one possible in the circumstances.

Table IV.

	Retention of calcium (mg. Ca per rat per day)							Calcium absorption (%)						
No. of rat	I	II			III			I	II			III		
F5	15·5	10·5	−3·5	—	−1·7	13·7	1·6	70	70	34	—	33	55	7
F6	23·4	21·7	12·1	13·8	27·6	24·6	—	87	95	94	95	95	72	—
F7	21·3	17·3	−3·2	—	3·7	17·8	12·3	71	86	30	—	36	49	36
F8	17·4	18·0	11·6	9·2	22·7	12·3	—	70	80	71	68	81	45	—
F21	11·8	−8·2	11·1	5·8	−0·5	18·1	5·7	42	9	81	70	25	49	20
F22	6·5	10·9	8·4	0·6	13·1	8·2	2·4	40	66	83	57	70	50	12

Period I. Preliminary.
" II. Irradiated ergosterol.
" III. Recovery.

Table V.

	Retention of phosphorus (mg. P per rat per day)							Phosphorus absorption (%)						
No. of rat	I	II			III			I	II			III		
F5	2·3	6·3	−11·9	—	11·9	14·3	10·4	85	85	62	—	81	80	65
F6	18·0	14·6	7·9	14·7	29·4	38·9	—	91	94	92	93	96	92	—
F7	17·1	10·8	−11·9	—	13·8	23·1	19·5	85	91	60	—	78	75	73
F8	15·8	12·9	1·3	7·8	15·0	11·3	—	83	87	80	81	80	73	—
F21	18·5	−6·7	14·5	2·9	11·0	12·0	2·4	81	56	90	85	66	77	57
F22	15·4	10·1	8·6	−5·2	19·6	5·8	2·4	78	87	90	80	85	66	60

Period I. Preliminary.
" II. Irradiated ergosterol.
" III. Recovery.

It will be seen that the balance of calcium during the period of irradiated ergosterol intake may fall quickly, as in rats F5 and F7, or more steadily as in F8, or very abruptly after several days on the diet as in the case of F22. The rapidity and extent of the fall ran closely parallel to the physical condition of the animal. Two rats developed negative balances while taking the

irradiated ergosterol. In most cases the amount of calcium retained was rapidly increased as soon as the excess vitamin was removed from the diet, but it is surprising to find that this improved retention was not maintained, and in five of the rats the positive balance was actually lower than normal after a few days on the normal diet. The phosphorus retention in general tended to follow similar lines.

The percentage of calcium apparently absorbed in the preliminary period was in most cases already so high that no very marked results from the irradiated ergosterol could be expected. There is however some evidence of increased absorption in all cases but one (F5), and it is interesting to note that with rats F21 and F22, where the initial absorption was lower, there is very definite evidence of the action of irradiated ergosterol in this respect. But the increased absorption was not maintained, and in every instance in the recovery period there was ultimately a definitely reduced percentage of absorption. A similar mechanism occurs in the case of the phosphorus, especially during the "curative" period.

It has to be remembered that these rats were losing weight. In order to get some idea of what effect, if any, loss of weight *per se* has upon calcium and phosphorus metabolism two male rats were given full synthetic diet in gradually decreasing amounts. The loss of weight experienced was 26 and 23 g. respectively, but the animals remained in good condition. The calcium excretion by the kidney ceased entirely, but in spite of this the retention declined rapidly (Tables VI and VII). The urinary phosphorus also became reduced in amount, roughly 50 %, and the amount retained decreased, rat F24 actually having a negative balance for the last three days. The absorption of both calcium and phosphorus was apparently much reduced by the low food intake, but again it must be emphasised that this may not be real, but may only mean that the path of excretion has been diverted from kidney to gut.

Table VI. *Calcium balance on low food intake.*

Mg. Ca per rat per day.

No. of rat	Intake	Faeces	Urine	Total	Retention	% absorption
F23	47·6	5·3	0·9	6·2	41·4	87
	26·5	9·2	0·9	10·1	16·4	65
	17·3	6·8	Nil	6·8	10·5	60
F24	50·8	14·2	1·1	15·3	35·5	72
	26·5	11·5	0·9	12·4	14·1	57
	17·3	9·9	Nil	9·9	7·4	43

Table VII. *Phosphorus balance on low food intake.*

Mg. P per rat per day.

No. of rat	Intake	Faeces	Urine	Total	Retention	% absorption
F23	96·7	6·7	57·5	64·2	32·5	93
	53·9	8·8	38·2	47·0	6·9	84
	35·2	5·2	26·7	31·9	3·3	85
F24	103·3	4·8	56·4	61·2	42·1	95
	53·9	10·3	41·7	52·0	1·9	81
	35·2	8·0	29·3	37·3	−2·1	77

Discussion.

Brown and Shohl [1930] have adequately stated the difficulties and dangers attendant upon any attempt to determine from urinary and faecal examinations whether the absorption has been altered or not. The figures given above must be accepted with reserve. It would appear from them that large doses of irradiated ergosterol tend at first to increase absorption of calcium and later to decrease it. We are on surer ground when dealing with retention, and here there is no doubt that excessive doses of irradiated ergosterol, combined with the resultant low food intake and loss of weight, reduce this amount very gravely. Recovery from the deleterious effects is evidently slow, for nine days after restoration to normal diet the balances were still gravely disturbed. During this period the calcium deposits are disappearing from the body, and the fact that the urinary excretion is declining, whereas the faecal elimination is unusually high, points to the probability that much of this deposited calcium is being eliminated by the gut rather than by the kidney. Whatever the effect of irradiated ergosterol may be on absorption it is clear that the hypercalcaemia and calcification of various tissues is not due entirely, if at all, to increased absorption, since the amounts retained in the body are less than is normally the case. The source of this calcium must be looked for elsewhere, and unlikely as it appears *a priori* that the action of large doses of a substance which normally aids calcification of the bones should be in the reverse direction, yet it is difficult to see where the deposited calcium comes from if not from the bones.

The phosphorus retention was greatly reduced as the result of the irradiated ergosterol, if anything more so than was that of the calcium. Yet regarded as a whole there was less disturbance of phosphorus metabolism, for the urinary phosphorus was not increased, or at most very slightly, and there was in general a quicker settling down to normal in the "curative" period. The reduced balance during the irradiated ergosterol period must not be attributed entirely to the effect of the hypervitaminosis, for loss of weight due solely to reduced food intake produces equally severe reduction in retention.

Summary.

1. Calcium and phosphorus retention in rats is decreased by excessive doses of irradiated ergosterol.

2. The urinary calcium is greatly increased, but not the phosphorus.

3. The faecal content of both calcium and phosphorus decreases, but not always in proportion to the intake; either the absorption is diminished in these cases, or increased amounts of absorbed material are being excreted by the gut.

4. Even graver disturbances in retention and absorption occur during the recovery period.

5. The low food intake and loss of weight characteristic of the hypervitaminosis probably contribute to some of these results.

6. The deposits of calcium found in certain tissues as the result of large doses of irradiated ergosterol cannot be accounted for on the grounds of increased absorption and retention.

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LXXIII. THE CHEMICAL ASPECT OF THE DRYING OF TIMBER.

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Introduction.

It is common knowledge that timber does not in general find its way into commercial usage until it has attained a moisture content approaching that which will be in equilibrium with the moisture of the atmosphere in which it will be used after conversion. In view of the high moisture content of freshly felled timber, the progress of drying under natural conditions must necessarily take considerable time, but despite this fact, and the economic issues which it involves, the question often arises as to whether air-drying of timber is to be preferred to the methods of kiln-drying which are now finding widespread application in the timber-using industry. In view of the belief existing in some quarters that certain beneficial chemical changes are associated with air-drying, prejudice has arisen from time to time against the practice of drying timber in kilns.

So far as it is known, no detailed chemical investigation of the effects of the drying of wood at moderate temperatures has hitherto been carried out, although throughout the literature of wood chemistry many references are to be found regarding the effect of high temperatures. It is pointed out by Hawley and Wise [1926, p. 197] that even at temperatures far below the destructive distillation point (approximately 280°) decomposition is indicated by the formation of small traces of acetic acid and methanol, probably as a result of hydrolysis. Under the conditions obtaining in a well regulated kiln-drying treatment of timber, however, there has been some doubt as to whether chemical changes of even a minor character are to be expected, in view of the fact that Hawley [1925] has stated that no chemical difference can be detected between the results of natural air-seasoning and artificial kiln-seasoning.

Since the value of wood as a structural material is dependent largely, if not entirely, on the nature of its skeletal substance, the composition of which varies but little from species to species, and since only such physical properties as colour, odour, taste, etc., are traceable to extractives, any chemical investigation on the effect of drying must in the first instance be confined to the effects produced on the major constituents of wood substance. The presence in wood, however, of substances characterised as extractives which may play a part in the seasoning process by acting on the major constituents

in some way must not be overlooked. It is for this reason, for example, that woods containing extractives of a notably acidic nature may react differently when dried under different conditions of temperature, as a certain amount of hydrolysis may be possible when high temperatures are employed. The wood of oak is known to contain small percentages of weak acids such as tannic acid, and it is conceivable that although small acid concentrations might only have a negligible effect on the major constituents of wood during air-drying, they might, none the less, have some effect when wood is dried at relatively high temperatures. As the amount of extractives in oak heartwood is invariably greater than that in the sapwood, it is conceivable that these might react differently to the same drying conditions. In the light of previous work [Hawley and Campbell, 1927] any increase in the alkali-solubility of wood after drying at relatively high temperatures would be indicative of chemical change of a hydrolytic nature. At the same time, however, there may be other chemical changes attendant on the drying of wood, which hitherto have escaped observation.

The present investigation has been undertaken therefore with a view to determining *de novo* whether any chemical changes accompany the drying of timber, and if so, whether any relationship exists between the changes attendant upon air-drying on the one hand, and artificial drying on the other.

The wood used was cut from coppice shoots of English oak of approximately 40 years' growth. After separation of the heartwood and sapwood, the material was quickly converted to sawdust and finally ground to pass an 80-mesh and be retained by a 100-mesh screen, the grinding parts of the mill being of acid-resistant steel. The analytical methods used were those recommended by Schorger [1926, p. 505] and Hawley and Wise [1926, p. 123] and the results throughout are expressed as percentages of the oven-dry weight of the original wood.

Experimental.

During conversion to sawdust the wood used in this investigation had undoubtedly dried to a considerable extent, but the 80–100 mesh sapwood and heartwood still retained 25·4 % and 27·2 % of moisture respectively. Moisture contents were determined both by the standard oven-drying method and the xylene method of Schwalbe [Schorger, 1926, pp. 505–506] with closely comparable results. These materials were analysed as soon as possible following conversion (Table I).

Table I. *Analysis of original wood.*

	Sapwood	Heartwood
Cold water-soluble	1·76	4·63
Hot water-soluble	4·16	9·79
Cellulose	56·46	53·01
Lignin	19·98	20·94
Methoxyl	6·36	6·92
Total pentosans	24·75	24·88
Pentosans not in cellulose	11·23	11·07
Pentosans in cellulose	13·52	13·81

Samples of both sapwood and heartwood were then extracted with 1 % NaOH solution, under the conditions laid down by Schorger [1926, p. 506] for the standard alkali-solubility determination of wood, and, after removal of the alkali by thorough washing with hot water, the residues were dried at 105° and analysed (Table II).

Table II. *Analysis of original wood after extraction with NaOH.*

	Sapwood	Heartwood
Loss on alkali extraction	19·0	22·92
Cellulose	56·96	53·87
Lignin	18·55	18·42
Methoxyl	4·70	4·23
Total pentosans	19·67	19·27
Pentosans not in cellulose	2·91	2·79
Pentosans in cellulose	16·76	16·48

The drying experiments were carried out as follows.

(i) *Air-drying.* Weighed samples of both sapwood and heartwood were placed in jars, covered with fine linen to prevent access to dust, and left in the laboratory with occasional mixing to dry for a period of 126 days. At the end of this time the moisture content of the sapwood had fallen to 13·0 % and that of the heartwood to 12·8 %.

No loss in dry weight due to air-drying was observed.

(ii) *Kiln-drying.* Weighed samples of sapwood and heartwood were subjected to a kiln-drying treatment as follows. The wood flour was in each case spread on a glass dish in a layer 1 inch deep and the following kiln-seasoning schedule, which was considered suitable for 1 inch oak boards, was applied.

Schedule employed in the kiln-seasoning of 80–100 mesh oak.

Temperature ° F.	Humidity %	Duration of treatment (days)	Total time (days)	Assumed moisture content %
115	75	2	2	30
120	65	4	6	25
125	55	5	11	20
130	45	5	16	15–10

The rate of initial warming of the sawdust was 9° F. per hour at a constant humidity of 75 %.

As a result of this treatment the moisture content of both the sapwood and heartwood fell to 7·5 %. Further, a loss of 0·2 % and 0·33 % of the dry weight of the original wood was observed in sapwood and heartwood respectively, and a decided darkening in colour was noted in each case.

(iii) *Oven-drying.* Weighed samples of sapwood and heartwood were placed in weighing bottles in a drying oven, and maintained at 105° for 200 hours. Pronounced darkening in colour took place in both sapwood and heartwood and the respective losses in weight of wood substance were 0·41 % and 1·12 %.

Analyses were now carried out on each of the residues remaining after the several drying treatments (Table III). Further, each residue was analysed

after alkali extraction under the same conditions as the extractions applied to the original wood (Table IV).

Table III. *Analysis of 80–100 mesh English oak wood after kiln- and oven-drying.*

Samples		Duration of treatment (days)	Loss due to treatment	Cold water-sol.	Hot water-sol.	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose	Pentosans in cellulose
Air-dried	Sap	126	Nil	1·52	3·59	57·57	21·22	7·06	23·76	8·90	14·86
	Heart		Nil	4·95	9·05	54·26	22·03	7·91	23·42	8·82	14·60
Kiln-dried	Sap	16	0·20	1·81	3·65	57·27	21·98	5·81	23·74	9·44	14·31
	Heart		0·33	4·37	9·17	52·53	22·47	5·49	23·11	8·89	14·22
Oven-dried	Sap	9	0·41	2·23	4·11	55·47	21·70	5·50	23·61	11·53	12·08
	Heart		0·12	3·94	9·84	52·00	23·60	4·76	23·78	11·62	12·17

Table IV. *Analysis of air-, kiln- and oven-dried wood after alkali extraction.*

Samples		Loss due to treatment	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose	Pentosans in cellulose
Air-dried	Sap	18·78	57·09	18·33	4·79	20·15	4·43	15·72
	Heart	23·41	53·70	17·95	4·38	19·73	4·79	14·94
Kiln-dried	Sap	18·88	56·32	19·48	4·74	19·36	3·73	15·63
	Heart	24·62	51·94	18·51	4·10	18·62	3·04	15·58
Oven-dried	Sap	19·41	54·52	17·62	4·35	18·94	3·82	15·12
	Heart	28·93	49·30	19·60	4·10	16·52	3·42	13·10

The effect of mild hydrolysis on the original wood.

A sample of the original heartwood was subjected to a hydrolytic treatment as follows. 100 g. of the material was incorporated with 2 litres of 1 % aqueous hydrochloric acid and maintained at 100° for 6 hours. After removal of the acid, thorough washing with hot water and drying at 105°, the residue was analysed (Table V).

Table V. *Analysis of 80–100 mesh English oak wood (heart) after hydrolysis with 1 % HCl for 6 hours at 100°.*

Loss on hydrolysis	33·43
Cellulose	42·44
Lignin	18·98
Methoxyl	4·06
Total pentosans	7·28
Pentosans not in cellulose	2·47
Pentosans in cellulose	4·81

Discussion.

Comparison of Tables I and III shows that, irrespective of the method of drying, the percentage amount of water-soluble material in the wood under investigation does not vary except within the limits of experimental error. Changes during drying, however, are apparent in the cellulose, lignin and pentosans.

So far as the cellulose is concerned this constituent exhibits a slight increase in amount in the passage from the green to the air-dried condition in both sapwood and heartwood. A similar though not quite so pronounced increase is noted in the cellulose content of kiln-dried sapwood, while the cellulose content of kiln-dried heartwood is slightly lower than that of the original wood. In the case of the oven-dried material depreciation of the cellulose content is observed to take place in both sapwood and heartwood. These slight variations in cellulose content are further seen to be accompanied by concomitant variations in the percentage amounts of furfuraldehyde-yielding complexes in the wood, for, while the total pentosan content exhibits a constant decrease in amount as a result of all three drying processes, the pentosan content of the cellulose increases on air- and kiln-drying, and decreases on oven-drying. The variations in the pentosan content of the cellulose agree very closely with the variations in the cellulose content itself, except in the case of oven-drying where the loss of pentosans in the cellulose is greater than that of the cellulose, and in kiln-dried heartwood where a slight increase in the pentosan content of the cellulose is accompanied by a decrease in cellulose content. Coupled with the fact that only slight losses in weight have been observed as a result of kiln- and oven-drying, the depreciations of cellulose and of pentosans in the cellulose indicate that kiln-dried heartwood and oven-dried heartwood and sapwood have undergone a certain amount of incipient hydrolysis, decomposition being most pronounced in oven-dried heartwood.

From the analytical data it is impossible to deduce to the fullest extent the true nature of the changes which take place in the furfuraldehyde-yielding complexes during drying. It is none the less evident, however, that definite changes take place in that portion of such bodies which in green wood is not associated with the cellulose, since, whether an increase or decrease in the pentosan content of the cellulose be observed, depreciation of the total pentosan content is a consistent result of drying by all three processes. The results therefore indicate that, of that portion of the furfuraldehyde-yielding complexes which is not associated with the cellulose in the original wood, part becomes associated with the cellulose during drying, and remains associated with it provided the drying conditions are not too severe, and part is transformed into something which ceases to yield furfuraldehyde on distillation with 12 % hydrochloric acid. It is probable that this latter portion becomes associated with lignin during drying, as this constituent exhibits a consistent increase in amount as a result of all three drying processes studied. Thus the question of the relationship between the lignin and pentosans of wood is again revived. From the conflicting experimental evidence available on this subject up till 1926, Hawley and Wise [1926, pp. 70–71] are unable to draw definite conclusions, but Jonas [1928] has recently discussed numerous theories of lignin constitution, and arrives at the conclusion that lignin is a condensed furfuran product containing the stable tetrahydrofurfuran ring.

Further light on the nature of the chemical changes attendant on the drying of wood is obtained only by a comparative study of the effect of alkali on green and dried woods respectively. Comparison of Tables I and II shows the effect of 1 % sodium hydroxide at 100° on the original green wood.

It can be seen that the pentosans and lignin are the components most susceptible to alkali attack, but the outstanding fact is that cellulose appears to be but little affected by alkali until it is observed that the pentosan content of the cellulose in alkali-extracted wood is higher than that of the cellulose isolated from the original wood. The explanation of this fact is not yet apparent, but from the results of research at present in progress it would appear that an increase in the pentosan content of the cellulose after alkali-extraction is characteristic of hardwoods.

Comparison of Tables II and IV shows that the total respective alkali-solubilities of green and air-dried wood are approximately the same, but closer examination of the data reveals that, while the alkali-solubility of the pentosans not in the cellulose is much less in air-dried wood than it is in the original wood, an increase in the alkali-solubility of lignin is noted as a result of drying. This points to the conclusion that the increase in the alkali-solubility of the lignin is attributable not so much to any effect of drying on the lignin nucleus as to the original susceptibility to attack by alkali of those bodies derived from the furfuraldehyde-yielding complexes which have become associated with it during drying. This is further borne out by the analytical data, which show that after air-drying the increase in alkali-solubility of the lignin is approximately equal to the increase in the percentage of that constituent which results from the drying treatment.

As a result of kiln-drying, the pentosans not in the cellulose are slightly more susceptible to alkali attack than the corresponding components of air-dried wood. In this case also it seems probable that the increased alkali-solubility of lignin is accounted for by the additions made to it during drying.

So far as oven-drying is concerned, the low cellulose yields recorded, coupled with the depreciation of the pentosan content of the cellulose, suggest that the severity of the treatment has induced a certain amount of hydrolysis. This is borne out by the data in Tables II and IV, which indicate that the pentosans in oven-dried wood are more susceptible to attack by alkali than the corresponding components in kiln- and air-dried wood. In conjunction with the fact that the cellulose in oven-dried sapwood is more resistant to alkali than the corresponding components in oven-dried heartwood, the experimental evidence favours the conclusion that prolonged heating of green wood at 105° has the effect of inducing hydrolysis, the results of which are more marked in heartwood than in sapwood.

The ease with which green oak heartwood can be hydrolysed is demonstrated by the data in Table V. Comparison of these with the data in Table I shows that the pentosans are by far the most susceptible to hydrolysis of all the principal wood components.

Conclusions.

From the foregoing considerations it is to be concluded that the drying of oak timber is associated with definite chemical changes in the furfuraldehyde-yielding complexes of the wood substance, which are responsible in their turn for alterations in the cellulose and lignin aggregates, as determined by standard analytical methods. In this respect close agreement has been proved between the nature of the chemical changes attendant on the processes of air- and kiln-drying, although the analytical data suggest that kiln-drying may induce very slight decomposition in heartwood. The effects of drying by continuous heating of wood at 105° for 200 hours are marked by a certain amount of decomposition which has been shown to be of a hydrolytic nature. It must further be concluded that the greater amount of hydrolysis noted in the heartwood is due to its higher content of extractives of an acidic nature. In this connection it is probable that the reason why it is found in practice that softwoods are much easier to dry at elevated temperatures than hardwoods, is that softwoods invariably contain less pentosans than hardwoods. The susceptibility of the pentosans of wood to hydrolysis has been amply demonstrated in previous work, but having regard to the evidence cited above it is conceivable that conditions which would be severe enough to cause marked depletion in the pentosans of a hardwood—with possible influence on the other major constituents, and probably the physical properties of the wood as a whole—might produce no marked detectable difference on a softwood, solely on account of the low pentosan content of the latter. While it is admitted that the effects of drying on the wood flour used in this investigation cannot implicitly be claimed for wood in its natural form, owing to purely structural considerations, the results show that in so far as its effect on wood substance is concerned, a well-regulated and suitable kiln-drying treatment should have no detectable deleterious effect. It is indicated, however, that if the conditions obtaining in the interior of a piece of wood in the process of kiln-drying should by any chance become so drastic as to approach the conditions obtaining in an oven-drying treatment, deleterious effects might reasonably ensue. This is all the more probable for the reason that if volatile acids cause the hydrolysis, their action would be of a more serious nature than that detected in the present investigation, in view of the fact that they could not volatilise so readily from the interior of a piece of wood as they could from the more discrete particles of a wood flour.

Summary.

1. The effects of air-, kiln- and oven-drying on both sapwood and heartwood of English oak have been examined in detail.

2. It is shown that air- and kiln-drying of wood are associated with chemical changes in the furfuraldehyde-yielding complexes of wood substance, to the extent that total pentosans are depreciated while the pentosan content of the cellulose is enhanced, and an increase in lignin is noted.

3. Oven-drying of wood is shown to result in an increase of lignin and a slight amount of hydrolysis of the carbohydrate components, which is more marked in the heartwood.

4. Reasons are given for concluding that well-regulated kiln-drying treatment of timber is strictly comparable in effect to ordinary air-drying. On the other hand, it is shown that if conditions in a kiln should become so drastic as to approach those obtaining in an oven-drying treatment, decomposition of the wood substance would be imminent.

5. It is suggested that the reason why softwoods can be kiln-dried with greater ease than hardwoods, is to be found in the fact that these invariably contain less pentosans than hardwoods, so that drying conditions which might be severe enough to hydrolyse the pentosans of wood would always affect hardwoods more than softwoods.

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LXXIV. STUDIES ON PECTIN.

V. THE HYDROLYSIS OF PECTIN.

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Introduction.

Many workers have shown that pectin exists normally as the methyl ester of the tetrabasic pectic acid. The carboxyl groups may be, and in fact are usually, only partially esterified. The first stage in hydrolysis is the process of de-esterification which involves no change in the six-membered ring. This may be brought about in the cold by alkali as dilute as $N/20$ or by warming in weak acid solution. Heating with stronger acid or alkali results in the destruction of a portion or all of the pectin. In other words, there is a rupture of the pectic ring. A knowledge of this process may be of value in three directions. In the first place, it is of importance to know the degree of stability of the pectin molecule and the critical concentrations of various reagents above which the ring is attacked. Such conditions can then be avoided in the preparation and handling of pectic substances. In the second place, information on the rate and route of hydrolysis may throw some light on the arrangement of the units and nature of the linkages in the ring, about which so little is as yet known. Nanji, Paton and Ling [1925] suggest that the linkages are "other than 1 : 6." While it is certain that group 6 does not participate, since the carboxyl groups of the galacturonic acid units are free, it is equally certain that group 1 is involved for pectin is a non-reducing body. The amylene oxide ring theory for sugars would suggest a 1 : 4 linkage, but this has yet to be proved. Further, it must not even be assumed that the linkage between each unit is of necessity of the same type. If suitable methods of attack could be devised, any difference in type or strength of linkage would become evident from the rate of hydrolysis and nature of the products formed. In the third place, a full knowledge of the degradation products given on hydrolysis might throw much light on the down-grade processes in the metabolism of the pectic substances. There is no definite information as to the nature of the intermediate and ultimate degradation products of pectin in the plant, the metabolic picture being thus incomplete. Pectin is not a final stable substance, as is cellulose or lignin, but more labile, since it disappears during the process of lignification.

O'Dwyer [1926] found that in unlignified tissues containing pectin the hemicellulose content is low, but in lignified tissues it is considerably higher and pectin is absent or practically so. She suggested [1928], therefore, that a stage in the process of lignification is the conversion of pectin to hemicellulose. Candlin and Schryver [1928] grouped both these substances as "polyuronides," and investigated the production of hemicellulose-like bodies by the alkaline hydrolysis of pectin. They claim that the conversion is a process of decarboxylation of the uronic acid groups brought about by the action of the alkali. They quote figures for the percentage loss of uronic anhydride content of pectin treated with various concentrations of alkali under different conditions, and hold the loss of uronic acid to be an index of decomposition. However, the extent of decarboxylation of the uronic acids could only be regarded as any measure of the degree of hydrolysis of the pectin, if the uronic acid groups were the most sensitive in the molecule and were instantaneously destroyed by the alkali as the ring was ruptured. This did not seem to be in agreement with the known properties of these acids and accordingly in the work to be described the degree of hydrolysis was determined by the only valid method possible, namely by the yield of calcium pectate. For comparison, the loss of uronic acid anhydride was also estimated as in Candlin and Schryver's work. Since it was hoped at the same time to obtain information as to the relative sensitivities of the various units to alkaline treatment, and the route of the degradation, the furfuraldehyde yield of the hydrolysis liquid was also determined.

Methods.

The methods employed were as follows.

(A) *Calcium pectate yield*; Nanji and Norman's [1928] modification of the Carré and Haynes [1922] method.

(B) *Total furfuraldehyde yield*; distillation with 12 % HCl, and precipitation of the furfuraldehyde as the phloroglucide, which is subsequently extracted with hot alcohol to remove impurities.

(C) *Total carbon dioxide yield*; Nanji, Paton and Ling's [1925] modification of the Lefèvre and Tollens [1907] method for uronic acids.

The pectin which was used throughout this work was a commercial sample prepared from Citrus fruits by the method of Wilson [1925].

The analytical figures obtained for this pectin calculated on 1 g. calcium pectate were:

furfuraldehyde yield (B)	17·72 %
carbon dioxide yield (C)	18·29 %
uronic anhydride content	73·16 %

It is seen that these figures do not precisely correspond with the theoretical. Calcium pectate should contain 69·7 % galacturonic acid anhydride, and yield approximately 19·5 % furfuraldehyde. However, commercial preparations,

and, indeed, most general preparations show a divergence from these figures. The difference is accounted for in this case by a content of anhydroarabinose rather below theoretical, *i.e.* 10·33 % instead of 14·25 %, and of galacturonic acid rather higher. It is not proposed to discuss this discrepancy here except to note that the Citrus-pectin employed by Candlin and Schryver in their work gave figures differing even more widely from the theoretical. Its content of uronic acid was 88·6 % and total furfuraldehyde yield 23·1 %. Their figures are not, however, calculated on calcium pectate obtained but on the crude preparation.

Calculation of results.

Knowing the composition of the original pectin solution, it was possible from the determinations mentioned above to calculate the pentose and uronic acid content of any degradation product present in the hydrolysis liquid. The calculations involved are given below.

(D) *Furfuraldehyde due to pectin*, $A \times \frac{17{\cdot}7}{100}$.

(E) *Uronic acids in pectin*, $A \times \frac{73{\cdot}16}{100}$.

(F) *Non-pectin uronic acid*, 4C–E.

(G) *Furfuraldehyde due to non-pectin uronic acid*, $F \times \frac{16{\cdot}66}{100}$.

(H) *Furfuraldehyde due to pentose not in pectin* (B–D)–G.

Whereas the calcium pectate yield (A) will be the true measure of degree of hydrolysis of the pectin, the non-pectin uronic acid (F) and furfuraldehyde due to pentose groups not in pectin (H) will show the resistance or otherwise of the uronic and pentose groups to the hydrolysing agent. If either is very low or nil it will indicate that that constituent is destroyed as rapidly as it is liberated from the pectin and is, in all probability, the point at which the pectin ring is susceptible to attack.

Experimental.

Alkaline hydrolysis.

The work of Candlin and Schryver [1928] indicates that considerable decarboxylation of pectin is effected by boiling with alkali of concentration $N/8$. They record 33 % decarboxylation in 30 minutes and 80 % decarboxylation in 4 hours. This strength of alkali was therefore employed in the first experiments. The various hydrolyses were carried out independently for ease and rapidity of operation.

50 cc. of a 0·4 % solution of Citrus-pectin were taken in a 250 cc. round-bottomed flask, and to it were added 5 cc. $N/10$ sodium hydroxide, which was the quantity necessary to convert the pectin to sodium pectate. The solution was brought to the boiling point and 20 cc. of 1·88 % NaOH were added. This brought the volume to 75 cc. and the final concentration of alkali to 0·5 %.

The liquid was boiled under a reflux for the requisite time, rapidly cooled, and exactly neutralised with *N* HCl. In certain cases in which the hydrolysis was nearly complete double quantities had to be taken.

For the furfuraldehyde estimation and uronic acid determination the acid concentration was brought up to 12·5 % by the addition of 42 cc. concentrated HCl. In the former case the distillation was then carried out in the usual way, while in the latter a stream of CO_2-free air was passed through the cold solution for half an hour to remove any carbon dioxide in solution.

Table I. *Alkaline hydrolysis of pectin.*

Expressed on 1 g. calcium pectate.

Time	Calcium pectate yield	Loss of calcium pectate %	Total furfurald. yield	Loss of furfurald. yield %	CO_2 yield	Loss of CO_2 yield %
			Hydrolysed with 0·5 % NaOH.			
Blank	1·0	—	0·177	—	0·183	—
5 min.	0·550	45·0	0·118	33·2	0·124	32·5
10 „	0·256	74·4	0·052	70·8	0·098	46·7
20 „	0·184	81·6	0·051	71·1	0·081	55·7
30 „	0·122	87·8	0·043	75·9	0·073	60·1
1 hour	0·057	94·3	0·038	78·9	0·061	66·9
3 hours	0·054	94·6	0·030	83·0	0·052	71·4
			Hydrolysed with 0·2 % NaOH.			
Blank	1·0	—	0·177	—	0·183	—
10 min.	0·305	69·5	0·103	41·8	0·142	22·2
20 „	0·235	76·5	0·089	49·4	0·136	25·9
30 „	0·207	79·3	0·069	60·9	0·132	28·1
1 hour	0·202	79·8	0·059	66·7	0·113	37·9
3 hours	0·156	84·4	0·057	68·0	0·064	65·2

Since hydrolysis with 0·5 % alkali appeared to be very rapid and extensive a similar series was carried out with 0·2 % NaOH. The results obtained in both series are quoted in Table I.

All the figures are the means of two or more closely agreeing determinations.

It was clear from the above figures that the hydrolysis of pectin or, rather, the rupture of the pectin ring is more rapid than the destruction of furfuraldehyde-yielding groups and than apparent decarboxylation, and does not seem to be conditioned by either of these. It is necessary to deduce the liberation of carbohydrate units either free or in simple combination in the hydrolysis liquid. Accordingly it was of importance to determine the behaviour of sugars in the presence of alkalis under similar conditions. The sugars tested were those present in pectin, namely, arabinose and galactose. In addition, lactose was similarly treated since it contains galactose in a combined form with glucose, the last mentioned being also treated independently. 0·2 g. of sugar in 25 cc. was boiled with 5 cc. of alkali of the necessary strength to give a 0·5 % or 0·2 % solution. The solution was exactly neutralised and the reducing power measured by the iodimetric method [Baker and Hulton, 1920]. The results are expressed as percentage loss of reducing power as measured by iodine absorption (Table II).

Table II. *Treatment of sugars with alkali.*

Sugar	Conc. of NaOH %	Percentage loss of reducing power 2 min.	4 min.	10 min.	20 min.	30 min.	1 hour
Arabinose	0·5	44·5	49·7	51·8	—	52·8	—
,,	0·2	38·2	43·5	49·7	—	53·9	—
Galactose	0·5	41·0	41·9	45·0	—	52 8	—
,,	0·2	27·5	32·6	40·5	—	46·1	—
Glucose	0·5	31·7	34·3	40·4	—	44·5	—
Lactose	0·2	+62·4	+37·3	+21·4	+13·9	+ 9·1	+7·2

Very rapid destruction of the sugars therefore takes place in the first few minutes of treatment with dilute alkali, and the reducing power observed at 10 minutes, which thereafter remains practically constant, was thought to be due not to sugars but to iodine absorption by the degradation products formed. That this is so was shown by treating the above sugars with 0·2 % alkali for 10 minutes, cooling and neutralising with acetic acid. Phenylhydrazine was then added under the usual conditions for preparation of an osazone, but none was obtained in the cases of the monosaccharides tested or even in the case of lactose.

It seemed therefore that units with free aldehydic groups would be destroyed within a few minutes of liberation, and that any uronic acid and pentose split off from pectin by rupture of the ring and found in the hydrolysis liquid must be linked with the aldehydic group shielded. In order that the quantity of such degradation products might be determined the methods of calculation previously described were applied to the figures quoted in Table I, and are given in Table III.

Table III. *Alkaline hydrolysis of pectin.*

Expressed on 1 g. calcium pectate.

Time	Calcium pectate yield (A)	Furfurald. due to pectin (D)	Non-pectin furfurald. (B–D)	Apparent total uronic content (4 C)	Pectin uronic content (E)	Apparent non-pectin uronic content (F)	Furfurald. from non-pectin uronic acid (G)	(H)
Hydrolysed with 0·5 % NaOH.								
Blank	1·0	0·177	—	—	0·732	—	—	—
5 min.	0·550	0·098	0·020	0·494	0·402	0·092	0·015	0·005
10 ,,	0·256	0·045	0·007	0·390	0·187	0·203	0·034	−0·027
20 ,,	0·184	0·033	0·018	0·324	0·134	0·190	0·032	−0·014
30 ,,	0·122	0·222	0·021	0·292	0·089	0·203	0·034	−0·013
1 hour	0·057	0·010	0·028	0·242	0·041	0·201	0·033	−0·005
3 hours	0·054	0·009	0·021	0·210	0·039	0·171	0·028	−0·007
Hydrolysed with 0·2 % NaOH.								
Blank	1·0	0·177	—	—	0·732	—	—	—
10 min.	0·305	0·054	0·049	0·569	0·223	0·346	0·058	−0·009
20 ,,	0·235	0·042	0·048	0·542	0·172	0·370	0·062	−0·014
30 ,,	0·207	0·037	0·032	0·526	0·151	0·375	0·062	−0·030
1 hour	0·202	0·036	0·023	0·452	0·148	0·304	0·051	−0·028
3 hours	0·156	0·028	0·029	0·254	0·114	0·140	0·023	0·006

This method of calculation, though based on justifiable theoretical knowledge, when applied to the figures in question showed certain marked discrepancies. In the tables above, the final column, headed (H), should in theory

give the furfuraldehyde due to pentose units not in pectin. It was found, however, that a negative result was obtained in practically every case. This could be due to one of two reasons. Either the total furfuraldehyde yields as obtained were uniformly low, or else the CO_2 yields were too high. It did not seem likely that any substance present in the hydrolysis liquid could lower the production of furfuraldehyde from the pentose units present on distillation with hydrochloric acid. It did, however, appear possible that the action of the alkali might produce substances, apart from uronic acids, which would give CO_2 on boiling with hydrochloric acid, although this reaction has been presumed to be specific.

The action of dilute alkalis on the polysaccharides is a field of carbohydrate chemistry as yet unexplored. The work of Lobry de Bruyn and van Ekenstein [1895, 1896, 1897] and of Nef [1907, 1910, 1914] and his co-workers on the effect of alkali on the monosaccharides has shown the very complicated nature of the reactions which take place. More recently Lloyd Evans [1929] has re-investigated these problems and extended the work to certain disaccharides. The results of all these workers have thrown much light on the constitutional relationships between the sugars. In practically all cases, however, they found it necessary to employ moderate temperatures so that the course of the reactions might be followed, and intermediary and transitory substances identified and estimated. However, differences in behaviour and quantities of end-products which were observed between individual sugars of the same series at a given alkalinity at lower temperatures were found to disappear as the temperature was raised. Evans [1929] states that at 75° the various hexose sugars yield practically identical amounts of oxidation products since they form a common 3:4-dienol. It is not unreasonable to assume that the reaction at 100° proceeds similarly to that described as taking place at 75°, but at a much increased rate. The hexoses are presumed to give in the first place an equilibrated mixture of three different dienolic forms, the 1 : 2, the 2 : 3, and the 3 : 4 isomers. Rupture then takes place at the double bonds of the several dienols and various degradation products are formed. In the cases just considered the 1 : 2-dienol gives an active pentose and hydroxymethylene, the 2 : 3-dienol, a tetrose and glycollaldehyde, and the 3 : 4-dienol, two molecules of glyceraldehyde. The pentoses and tetroses similarly give dienolic forms which on rupture yield the same products. Subsequent oxidation results in the production of CO_2 from hydroxymethylene, and in part from glycollaldehyde and glyceraldehyde. Glycollaldehyde at the same time gives oxalic acid, or may give some glycollic acid. Glyceraldehyde is converted into pyruvic aldehyde which in turn gives lactic acid or may give acetic and formic acids.

The extent to which this process is modified by linkage of the sugar is, as yet, not fully determined. The only linked hexose product which has been investigated under alkali treatment is maltose. Evans and Benoy [1930] have shown that alkaline solutions of this sugar contain an equilibrated system

similar to that observed with the hexose sugars, the components of the system being the 4-glucosido-glucose dienols and their fission products. In the case of the treatment of pectin with alkali the processes of hydrolysis and dienolic fission must go on simultaneously, and upon the precise point or points of rupture of the ring will depend the course of the subsequent fission. In addition to the products given above, derivable from the hexose and pentose units, the presence of galacturonic acid renders other products likely. These it is proposed to investigate later. A 3 : 4 dienol of galacturonic acid would give one molecule of glyceraldehyde and an aldehyde acid, giving in turn probably pyruvic, glyoxylic or oxalic acid.

The possible ultimate fission products of pectin on alkaline treatment are therefore, formic, acetic, oxalic, lactic, glycollic, glyceric, glyoxylic and pyruvic acids. It seemed not unlikely that one or more of these might yield CO_2 on treatment with 12 % HCl, and its presence in the hydrolysis liquid be responsible for the unaccountably high figures for CO_2 yield obtained in the experimental work just described. Samples of each were therefore boiled with HCl under the usual conditions, and the CO_2 evolved, if any, estimated (Table IV).

Table IV. *CO_2 evolution from certain acids on treatment with 12 % HCl.*

Acid	CO_2 evolved %	Acid	CO_2 evolved %
Formic acid	Trace	Glycollic acid	Nil
Acetic acid	Trace	Glyoxylic acid	Nil
Oxalic acid	0·42	Glyceric acid	Nil
Lactic acid	0·08	Pyruvic acid	5·4

It will be seen that among these only pyruvic acid was found to yield any quantity of CO_2. It was not, however, found possible to characterise this acid in the hydrolysis liquid, owing to the presence of other decomposition products, but theoretical considerations and this indirect evidence point to its formation in this process. Oxalic acid is known to be formed in some quantity and undoubtedly must contribute to the yield of CO_2.

For comparison a hexose and a disaccharide were treated with 0·2 % alkali for various periods and after neutralisation 12 % HCl was added and the CO_2 obtained on distillation measured (Table V).

Table V. *Hydrolysis of sugars with 0·2 % alkali.*

Sugar	Time of heating min.	CO_2 yield %
Glucose	2	0·98
,,	5	0·82
,,	15	0·70
,,	30	0·73
Lactose	30	1·12

In the method of calculation described earlier, CO_2 from the dienolic fission products would be calculated as uronic acid anhydride thus giving for this, as was suspected, a high and unreliable figure. Since it is not possible

satisfactorily to remove these fission products from the hydrolysis liquid, it is patent that this method of calculation is inapplicable for the purpose of throwing light on the composition of the higher degradation products of pectin.

Certain other methods of hydrolysis were tested, since it was supposed that these disadvantages might be confined to alkaline treatment. Acid hydrolysis seems to have received even less attention than hydrolysis by alkali. Ehrlich [1917] states that mild acid hydrolysis by boiling for several hours with 1 % oxalic acid yields an araban and a monobasic acid, galactose-galacturonic acid, $C_{12}H_{20}O_{12}$. Complete acid hydrolysis by boiling for 2–3 hours with 1 % HCl is stated to go further and to give free galactose and galacturonic acid. Hydrolyses were therefore carried out employing these two reagents under precisely similar conditions as described for the alkaline treatments. Since it was necessary to remove the oxalic acid before the uronic acid determinations were made, a slight excess of calcium chloride was added and the precipitated calcium oxalate filtered off. The washing of the alcohol precipitate in the pectin estimation with acid alcohol had to be carried out with great care till the oxalate test was negative. The results obtained, together with the calculations therefrom, are given in Table VI.

Table VI. *Hydrolysis of pectin.*

Expressed in g. per 1 g. calcium pectate.

Time hours	Calcium pectate yield (A)	Total furfurald. yield (B)	CO_2 yield (C)	Furfurald. due to pectin (D)	Non-pectin furfurald. (B–D)	Apparent total uronic content (4 C)	Pectin uronic content (E)	Apparent non-pectin uronic content (F)	Furfurald. from non-pectin uronic acid (G)	(H)
				Hydrolysed with 1 % HCl.						
Blank	1·0	0·177	0·183	0·177	—	—	0·732	—	—	—
1	0·549	0·255	0·210	0·097	0·158	0·840	0·401	0·439	0·073	0·085
3	0·155	0·242	0·208	0·207	0·215	0·832	0·113	0·719	0·120	0·095
5	Nil	0·231	0·128	—	0·231	0·512	—	0·512	0·085	0·146
				Hydrolysed with 1 % oxalic acid.						
Blank	1·0	0·177	0·183	0·177	—	—	0·732	—	—	—
4	0·635	0·206	0·191	0·112	0·094	0·764	0·465	0·299	0·050	0·044
8	0·325	0·282	0·190	0·058	0·224	0·760	0·238	0·522	0·087	0·137
12	Nil	0·210	0·190	—	0·210	0·760	—	0·760	0·126	0·084

An examination of these results showed two features of interest. In the first place there was observed in both cases a definite and considerable increase in total furfuraldehyde yield which can be accounted for with little difficulty. The action of the dilute acid on the galacturonic acid is in part at least a simple decarboxylation. From a unit which yields 16·66 % of its weight of furfuraldehyde is given a pentose unit which yields nearly 60 % of furfuraldehyde. The observed increase of furfuraldehyde yield is therefore due to the formation of pentose groups by simple decarboxylation of the uronic acids. In the second place an increase in carbon dioxide yield was observed in both

cases despite the fact that uronic acids have undergone decarboxylation to pentoses. It is seen, therefore, that the theoretical method of calculation employed is again invalid, and it is necessary to postulate in these cases also the production of non-uronic degradation products yielding CO_2 on treatment with 12 % HCl. It is not possible to speculate on the nature of these degradation products since so little is known as to the course of mild acid scission of polysaccharides.

A final hydrolysis was carried out by heating a pectin solution alone in an autoclave at 1 atmosphere for 1 hour. Considerable acidity is developed, and the final p_H was approximately 2·0. It is this development of acidity which prevents the setting of agar media containing pectin. The results are given in Table VII. Once again an increase in total furfuraldehyde yield was observed which is only in part balanced by the loss of uronic acid, necessitating again the postulation of the production of CO_2-yielding degradation products.

Table VII. *Hydrolysis of pectin solution in autoclave at 1 atmos.* (*Final* $p_H = 2{\cdot}0$.)

Expressed in g. per 1 g. calcium pectate.

Time	Calcium pectate yield (A)	Total furfurald. yield (B)	CO_2 yield (C)	Furfurald. due to pectin (D)	Non-pectin furfurald. (B–D)	Apparent total uronic content (4 C)	Pectin uronic content (E)	Apparent non-pectin uronic content (F)	Furfurald. from non-pectin uronic acid (G)	(H)
Blank	1·0	0·177	0·183	0·177	—	—	0·732	—	—	—
1 hour	0·904	0·219	0·172	0·160	0·059	0·688	0·687	0·001	—	0·059

Preparation of hydrolysis product.

Candlin and Schryver [1928] prepared two polyuronide products by the alkaline hydrolysis of pectin. These products both had a low uronic anhydride content, which was not surprising, since the hydrolysis was continued for 4 hours, a period which, in view of the figures given in Table I, would seem to be rather too long. Accordingly a hydrolysis was carried out for a shorter period. 20 g. of Citrus-pectin were dissolved in 1000 cc. water and NaOH added to make a concentration of 0·5 %. The whole was boiled for 1 hour under a reflux and exactly neutralised with hydrochloric acid. The solution was concentrated under reduced pressure to 200 cc. and made acid with hydrochloric acid. The precipitate which formed was filtered off, and will be referred to as the residue. The filtrate was poured into two volumes of 95 % alcohol and, after standing overnight, filtered. The precipitate was redissolved in dilute acid and reprecipitated with alcohol, and finally dried in graded strengths of alcohol. The residue was treated similarly. The yield of product was about 4 g. and of residue rather less.

The furfuraldehyde and CO_2 yields were determined on both the residue and the product.

	Residue %	Product %
Ash	6·67	16·47
CO_2 yield (ash-free)	18·27	14·31
Uronic anhydride (ash-free)	73·08	57·24
Furfurald. yield (ash-free)	17·24	14·68

It is seen that the composition of the residue closely approximates to that of the original pectin, and it is therefore unchanged pectic acid.

From the figures given above it is possible to calculate the composition of the hydrolysis product. The composition of the products obtained by Candlin and Schryver [1928] has similarly been calculated from their results, and is given for comparison in Table VIII.

Table VIII. *Composition of alkaline hydrolysis products of pectin* (%).

	(Norman and Martin) Citrus-pectin	(Candlin and Schryver) Citrus-pectin	(Candlin and Schryver) Onion-pectin
Uronic anhydride	57·24	37·25	21·55
Total furfurald.	14·68	17·40	8·43
Furfurald. due to uronic anhydride	9·53	6·21	3·59
Furfurald. from pentose	5·15	11·19	4·84
Anhydropentose	9·64	20·84	9·06
Anhydrohexose	33·12	41·91	69·39

Like the products obtained by Candlin and Schryver [1928] the hydrolysis product in this case was non-reducing, but hydrolysable by N H_2SO_4 into reducing substances. Arabinose was characterised by the diphenylosazone and galactose by the production of mucic acid.

General discussion.

It is not possible from the results presented above to come to any conclusions as to the arrangement of the units in the pectin molecule, or as to the type of linkage involved. Nor is one able to point to any single linkage or unit as being peculiarly susceptible to attack by a particular hydrolysing agent, and being therefore the point at which rupture of the ring takes place. A theoretically sound method of calculation for determining the composition of the polyuronide derivatives from pectin breaks down both in the case of alkaline and acid hydrolysis, owing to the production of simpler degradation products which interfere with the determination of the uronic carboxyl groups.

The susceptibility of pectin at 100° to alkali as dilute as $N/20$ is particularly noteworthy since it indicates that the carbohydrate linkages are of a type apart from those found in some other polysaccharides, such as cellulose and starch. Since the production of simple degradation products, yielding CO_2 on treatment with 12 % HCl, can be accounted for by a process of dienolic fission following upon the rupture of these linkages, it seems likely that hydrolysis follows this course. It is clear that the CO_2 yield cannot be taken as a measure of decarboxylation and still less of hydrolysis of pectin as was done by Candlin and Schryver [1928]. They quote the percentage loss of uronic

anhydride calculated from CO_2 yield from pectin treated with different concentrations of alkali for various times and at several temperatures. The error in their figures due to the presence of dienolic fission products will vary with the conditions and will be considerable, as is seen in the work described above. Where it is possible to compare the apparent decarboxylation produced by the different concentrations of alkali employed by them, it appears that the lower concentrations are more effective, and in fact these workers comment on this. It seems likely that with the higher concentrations of alkali the enolic fission products are more completely degraded than with the lower, and so do not give CO_2 or as much CO_2 on treatment with HCl.

The principles underlying the mild acid hydrolysis are even more obscure, and, apart from the observation that pentose units are formed by simple decarboxylation of uronic groups, it is difficult to make further assertions. That there is simultaneously the production of non-uronic units giving CO_2 on treatment with HCl is clear from the results, but there is no evidence as to the nature of these.

It seems that valid information as to the nature of the polyuronide degradation products can only be obtained by the tedious process of actual isolation at various stages. Even then it is probable that the products isolated may be mixtures of derivatives with similar properties. That polyuronides similar in properties to the natural hemicelluloses may be obtained by the alkaline hydrolysis of pectin, as claimed by Candlin and Schryver [1928], is certain, but the process, as is shown above, is something more than simple decarboxylation, and, furthermore, can hardly be imagined as taking place in the plant. Norman [1929] has suggested that the production of hemicelluloses and of pectin in the plant is due to the mild oxidation of hexosan units. Recently Norman and Norris [1930] have shown that decarboxylation may be effected by a process of mild oxidation, thus lending some support to this theory. It is possible that enolic fission may play a part in the metabolism of these substances. Nef [1914] stated that dienols did not exist in any solution containing less than $N/30$ alkali, but this has recently been disproved by Evans *et al.* [1928] who show that limited dienol formation takes place in solutions of p_H less than 7·0. By employing copper acetate as a mild oxidant they were able to isolate some of the characteristic products of dienolic fission. It is conceivable therefore that these two methods of mild oxidation may be jointly responsible for the degradation in the plant of pectin to hemicelluloses. Oxidation of linked units may take place as in the hydrogen peroxide work of Norman and Norris [1930] and of unlinked units by limited oxidative dienolic fission owing to the p_H of the cell sap.

SUMMARY.

1. Hydrolyses of pectin have been carried out at 100° with 0·5 % and 0·2 % alkali. The yields of calcium pectate, furfuraldehyde, and CO_2 were estimated at intervals.

2. A rapid hydrolysis was observed in both cases; the rupture of the pectin ring proceeding faster than the destruction of furfuraldehyde-yielding groups and apparent decarboxylation.

3. When a calculation was made to determine the nature of the immediate degradation products of the pectin, certain discrepancies became apparent. The CO_2 yield was higher than could be accounted for from the uronic acids present.

4. On theoretical grounds the presence of certain dienolic fission products of sugars was suspected, and the CO_2 yields of these were tested. Pyruvic acid was found to yield a considerable amount of CO_2, and oxalic and lactic acids a little. There is therefore indirect evidence for their presence in the alkaline hydrolysis liquid.

5. In the course of mild acid hydrolyses an increase in furfuraldehyde yield was observed, due to simple decarboxylation of uronic acids with the production of pentose. Despite this decarboxylation, an increase of CO_2 yield was noted, owing to the formation of non-uronic degradation products of unknown composition, yielding CO_2.

6. A polyuronide derivative of pectin containing 57 % uronic anhydride was prepared by alkaline hydrolysis of pectin, and its composition was determined.

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LXXV. THE PHOSPHORUS DISTRIBUTION IN BLOOD AND THE CALCIUM AND PHOSPHORUS EXCRETION DURING HYPERVITAMINOSIS D.

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(*Received May 1st, 1930.*)

It has been shown by several workers [Harris and Moore, 1928, 1, 2; 1929; Kreitmar and Moll, 1928; Pfannenstiel, 1927] that the feeding of doses of irradiated ergosterol far in excess of the therapeutic dose produces well-marked metabolic disturbance, a condition which has been called hypervitaminosis D. This condition is characterised by an extensive deposition of calcium salts in various organs of the body and is accompanied by an increased blood-inorganic phosphate [Harris and Stewart, 1929]. Hess and Lewis [1928] also observed signs of high blood-calcium and -inorganic phosphate in clinical cases of rickets, which were receiving 2·5–5 mg. of irradiated ergosterol per day. As phosphorus occurs combined in several different forms in blood, it was thought of interest to investigate whether the increase in inorganic phosphate, which in this condition can be maintained over a long period, produces any change in the blood content of acid-soluble organic phosphorus compounds. The work reported here was carried out to investigate this point.

In these experiments, the technique of Kay and Robison [1924] has been employed, and the following four types of phosphorus compounds have been studied.

(i) The inorganic phosphate.

(ii) The acid-soluble organic phosphorus compounds, almost all of which are present in the corpuscles. They are estimated by difference between total acid-soluble phosphorus and the inorganic phosphate.

(iii) That fraction of (ii) which is hydrolysed by Robison's bone-phosphatase, "the hydrolysable fraction."

(iv) That fraction of (ii) which is not hydrolysed by bone-phosphatase, "the non-hydrolysable fraction."

These four fractions, together with the "lecithin"-phosphorus, which has not been estimated, between them contain the whole of the phosphorus in blood.

[1] A short account of this work appeared in *Chem. Ind.* (1930), **49**, 237.

Experimental.

Twelve Dutch rabbits of approximately the same size and age were used in this work. At the beginning of the experiment they were about 3 months old. At the end they were fully grown and adult. The data therefore are for both growing and adult rabbits. They were given a mixed diet of bran and roots. No attempt was made either to increase or to limit the intake of calcium or phosphorus as was done in the experiments of Hess, Weinstock and Rivkin [1928]. After a control period lasting a month, the rabbits were divided into four groups and the following additions made to their diets.

A. 0·5 cc. arachis oil (2 rabbits);

B. 0·5 cc. arachis oil, containing 10 mg. irradiated ergosterol (4 rabbits);

C. 0·5 cc. arachis oil, containing 10 mg. ergosterol (2 rabbits);

D. no addition (4 rabbits).

The irradiated ergosterol used was "B.D.H.—irradiated in alcohol." The oil was fed each day by means of a pipette. The rabbits were weighed at intervals, the onset of hypervitaminosis being shown by a cessation of growth followed in time by a loss of weight.

Both during the control period and after feeding had started small samples of blood were withdrawn at intervals from the ear. The experimental feeding always followed bleeding so as not to excite the animals. The blood samples were received into paraffined crucibles, containing a minimal amount of potassium oxalate. The volume of corpuscles was estimated by haematocrit, but as it varied very little from the average figure of 34 % no corrections have been made and all results are expressed as mg. per 100 cc. of whole blood. 2 cc. were measured by pipette and delivered into 5 cc. of 25 % trichloroacetic acid. After standing for $\frac{1}{4}$–$\frac{1}{2}$ hour they were filtered into 25 cc. volumetric flasks, neutralised to phenolphthalein and made up to the mark. When not estimated immediately the filtrates were kept in the ice-chest. 5 cc. were taken for estimation of inorganic phosphate. Total acid-soluble phosphorus was estimated on 1 or 2 cc. by incineration with strong sulphuric acid and Merck's perhydrol. For the estimation of the hydrolysable fraction, 5 cc. were treated with 1 cc. of 5 % bone extract and adjusted to p_H 9. They were incubated for 24 hours (which was found to be the most suitable time) at 37° in the presence of a little chloroform. 1 cc. of *N* sulphuric acid was added to precipitate the proteins and, after standing overnight in the ice-chest, the precipitate was filtered off and the inorganic phosphate estimated, a correction being applied for the inorganic phosphate added in the bone extract. All estimations were carried out in duplicate by the method of Briggs [1924].

The results are shown in Fig. 1. The three types of control A, C and D, have been treated together as they behaved in the same manner. It will be seen that, although the inorganic phosphate was raised by nearly 100 % at one time when the rabbits were young, and at another by nearly 50 % when

the rabbits were adult, no significant change in the content of acid-soluble organic phosphorus compounds was detected. The hydrolysable and non-hydrolysable fractions are shown in Table I. It can be seen that there are no significant differences between the control and experimental periods.

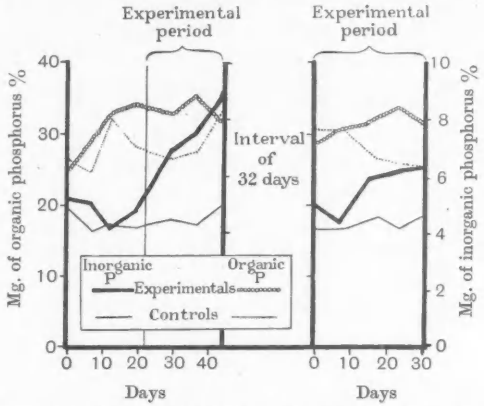

Fig. 1. Inorganic and acid-soluble organic phosphorus of control and experimental animals.

Table I. *Average values during control and experimental periods. All values mg. phosphorus per 100 cc. whole blood.*

Rabbit No.	Total organic		Hydrolysable		Non-hydrolysable	
	Control period	Experimental period	Control period	Experimental period	Control period	Experimental period
3	33·48	31·42	6·04	4·84	27·06	26·58
4	29·44	29·80	5·80	6·15	23·64	23·65
6	32·15	32·05	5·76	5·41	26·39	26·64
7	30·82	31·90	5·80	5·67	25·02	26·13

Calcium.

It has already been mentioned that Hess and Lewis [1928] observed hypercalcaemia in clinical cases of rickets receiving 2·5–5 mg. of irradiated ergosterol per day. Hess, Weinstock and Rivkin [1928] also observed that, in adult animals with a low serum-calcium and a very low intake, the level was rapidly raised to normal by large doses of irradiated ergosterol. Harris and Stewart [1929] reported a very slight rise in the adult rabbit and a 25 % rise in the case of rats. It was therefore thought desirable to carry out some estimations of serum-calcium, and I am greatly indebted to Miss E. Watchorn for being so kind as to do these. The method used was that of Kramer and Tisdall [1921]. The results are shown in Table II. It will be seen that the experimental animals have, on the whole, a higher serum-calcium than the controls, although the control values are rather low for rabbits. It should be mentioned that the experimental animals were in a condition of hyper-

vitaminosis during the whole period and at that time were adult. Rabbit No. 7, when killed on March 11, had a serum-calcium of 21 mg. per 100 cc. and an inorganic phosphate of about 8 mg. The symptoms were very severe and the animal had practically stopped eating and had fallen in weight from 3 lbs. to 2 lbs. in 10 days. Extensive calcification was visible to the naked eye in the aorta and kidneys, which were enlarged. Rabbit No. 6, showed the same symptoms and had a serum-calcium of 18·8 mg. per 100 cc. on March 30. It would seem that the rise in serum-calcium is a somewhat slower process than the rise in inorganic phosphate.

Table II. *Serum-calcium; mg. per 100 cc.*

	Experimental				Controls						
	Arachis oil + irradiated ergosterol				Arachis oil		Arachis oil + ergosterol		No addition		
Rabbit No.	3	4	6	7	1	2	10	12	5	8	9
Feb. 12	—	—	13·9	15·2	—	12·9	12·7	—	12·6	—	—
„ 19	14·4	13·1	—	—	—	—	—	10·1	—	10·1	10·8
Mar. 4	13·9	11·4	13·7	13·3	—	—	—	—	—	—	—
„ 11	—	—	—	21·0	—	—	—	—	—	—	—
„ 30	—	—	18·8	—	—	—	—	—	—	—	—

Table III. *Daily urinary output of calcium in mg.*

	Experimental				Controls						
	Arachis oil + irradiated ergosterol				Arachis oil		Arachis oil + ergosterol		No addition		
Rabbit No.	3	4	6	7	1	2	10	12	5	8	9
Feb. 15	37	—	—	83	—	34	Trace	—	Trace	—	—
„ 23	67	—	—	—	—	11	—	—	—	—	—
„ 24	51	—	—	29	—	—	Trace	—	Trace	—	—
„ 26	—	37	Trace	—	—	—	—	Trace	—	Trace	—
„ 27	—	—	72	—	Trace	—	—	—	—	—	—
Mar. 1	—	45	161	—	Trace	—	—	Trace	—	Trace	—
„ 3	—	108	85	—	Trace	—	—	Trace	—	Trace	—
„ 6	—	83	39	—	Trace	—	—	Trace	—	19	—
„ 7	—	130	—	—	—	—	—	16	—	53	—

Urinary calcium and phosphate.

During the latter part of the experiment urine was collected from control and experimental animals. The first fact which emerges is the absence of the diuresis which Hoyle and Buckland [1929] reported with rats. Fig. 1 shows that, if anything, the daily volumes are slightly less, which is probably accounted for by the decreased intake of food and hence water. Fig. 2 shows that the daily excretion of inorganic phosphate (estimated by the uranium acetate titration method) is consistently higher than in the controls. The urines were very alkaline (about p_H 8·5) as were also those of the controls, but, unlike the latter, they contained a white precipitate of calcium salts. Calcium estimations were again carried out by Miss Watchorn. The results (Table III) show a large excretion of calcium salts by the kidney, from a mere trace in the case of the controls to as much as 100 mg. per day

in the experimental animals, which were in a condition of hypervitaminosis during the whole period. It is interesting that a sample of urine (about 25 cc.) taken from the bladder of one of the animals after killing contained 156 mg. of calcium per 100 cc.

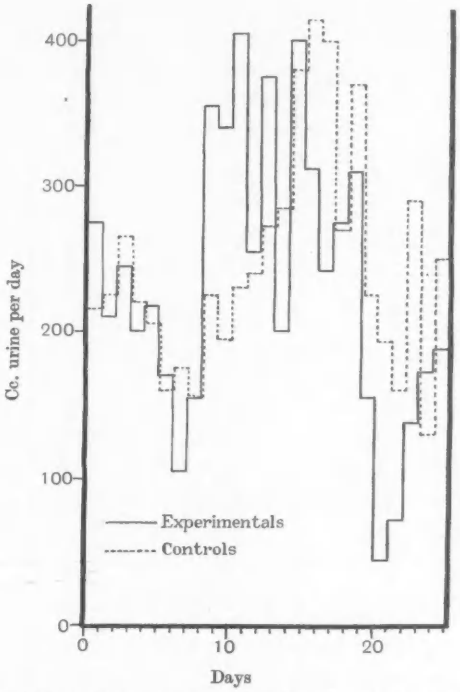

Fig. 2. Daily urinary volumes of control and experimental animals.

Recovery from hypervitaminosis.

Feeding of irradiated ergosterol to one animal was discontinued on February 26. A fortnight later, however, this animal was still excreting large amounts of calcium and a high level of inorganic phosphate by the kidney. Even on April 2 the urine contained much calcium and phosphate. When the animal was killed and examined on this date, calcification in the aorta was entirely absent and the kidneys appeared normal. It would appear from the urine, then, that recovery is very much slower than the rapidly falling blood-inorganic phosphate would lead one to suppose. On the other hand, the serum-calcium was still high, 16·6 mg. per 100 cc., while the blood-inorganic phosphate was 4 mg. per 100 cc.

Discussion.

It was shown by Wigglesworth and Woodrow [1923], that, when the blood-inorganic phosphate was raised by ingestion of phosphate, no increase in the organic esters took place. On the other hand, Haldane, Wigglesworth and Woodrow [1924] and Kay [1924] observed a rise in blood-inorganic phosphate in acidosis, accompanied by a fall in the content of organic acid-soluble phosphorus compounds, which Kay traced to the non-hydrolysable fraction. He suggested that this fall was necessary to help to neutralise the free acid, and there was an increased phosphate excretion by the kidney. Conversely, alkalosis caused a diminution in free phosphate. In the experiments reported

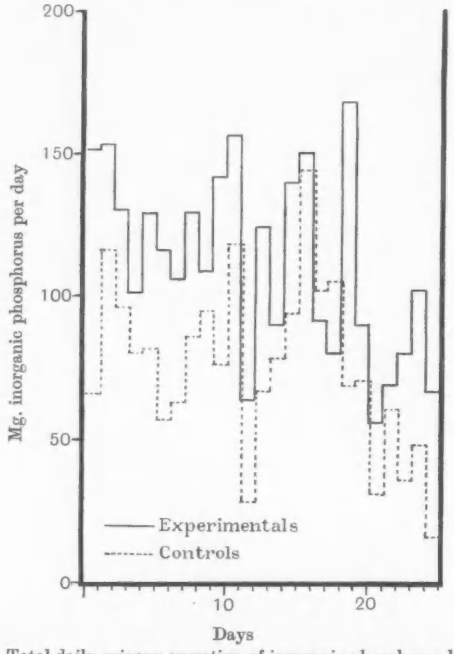

Fig. 3. Total daily urinary excretion of inorganic phosphorus by control and experimental animals.

here the p_H of the plasma was not determined, but there was no sign of ketosis in the urine, in spite of the increased phosphate excretion by the kidney. It seems unlikely that the relationship between the inorganic and organic phosphorus compounds of blood can be a simple one, because, if such were the case, a change in one, unaccompanied by reaction changes, should produce a change in the other. A probable explanation of the findings reported here is to be found in the work of Jost [1927] and of Martland, Hansmann and Robison [1924]. Jost showed that by far the greater portion of the acid-soluble organic esters of the red corpuscles consists of a diphosphoglyceric

acid, and that glycolysis in blood is associated with a synthesis of this ester. Martland, Hansmann and Robison showed the presence of an esterase in blood which attacks the organic esters under suitable conditions of p_H. Both these processes appear to be independent of the inorganic phosphate level within the limits obtaining in the blood, and hence no change in the organic esters was detected in the experiments described here. The system, in fact, is somewhat analogous to that obtaining in the synthesis and hydrolysis of hexosephosphate in alcoholic fermentation. Any accumulation of organic phosphorus esters in the plasma is probably prevented by hydrolysis in the kidney, which Eichholtz, Robison and Brull [1925] showed to be able rapidly to break down added esters.

With regard to the origin of the increase in blood-inorganic phosphate and calcium in hypervitaminosis there is no certain knowledge. It has been suggested that one of the functions of vitamin D is to regulate absorption from the gut, and the increased phosphate and calcium excretion by the kidney might suggest that this is the case. On the other hand, Hess, Weinstock and Rivkin [1929] suggest that the calcium comes from the bones. Total balance experiments in rats carried out by Light, Miller and Frey [1929], Brown and Shohl [1930] and Watchorn [1930] seem to support this view. Similar experiments under different levels of calcium and phosphate, which are being carried out in this laboratory at the present time, may serve to throw additional light on the two theories outlined above. In the experiments reported here, however, histological examination of the bones by Mr J. R. M. Innes hardly suggested that the calcium came from the bones, as these were hard and well calcified.

Summary.

1. The increase in blood-inorganic phosphate, which accompanies hypervitaminosis D, does not lead to any change in the content of organic acid-soluble phosphorus compounds.

2. A rise in serum-calcium during hypervitaminosis is confirmed.

3. Hypervitaminosis produces an increase in inorganic phosphate excretion by the kidney, probably with a slight decrease in the daily urinary volume.

4. There is a greatly increased excretion of calcium by the kidney.

5. Animals recovering from hypervitaminosis D maintain a high phosphate and calcium excretion by the kidney, when, apparently, other symptoms have disappeared.

My thanks are due to Mr J. B. S. Haldane and to Dr L. J. Harris for their interest and advice in this work, and to Miss E. Watchorn for carrying out the calcium estimations, and also for much advice. The animals were in the care of Mr F. Johnson, to whom I am extremely grateful.

This work was done during the tenure of a grant from the Department of Scientific and Industrial Research, for which my thanks are due.

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LXXVI. OXIDATION-REDUCTION POTENTIALS OF CULTURES OF *C. DIPHTHERIAE*. I.

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Many bacteria, particularly facultative anaerobes, may be cultivated under widely different environmental conditions. It seems probable that the varying degrees of access to atmospheric oxygen of cultures must affect the metabolism of organisms and their general biological behaviour.

The bleaching of methylene blue is a familiar example of the reducing conditions developed in bacterial cultures during growth, but the disadvantages and uncertainties attaching to the use of dyes previously mentioned [Hewitt, 1930] necessitate the use of a more accurate method of following oxidation-reduction processes in bacterial cultures.

Reducing conditions established during growth may be followed, therefore, by the fall in potential of bacterial cultures. This has been observed by Gillespie [1921], Cannan, Cohen and Clark [1926] and Coulter and Isaacs [1929]. Fildes [1929] found that a limiting reducing potential was necessary for the germination of tetanus spores. In a previous communication [Hewitt, 1930] it has been shown that conditions of oxygen supply have a very marked effect on the potentials developed in cultures of haemolytic streptococci.

C. diphtheriae may be cultivated on the surface of solid media with free access to oxygen, in liquid media with a poorer supply of oxygen, or even anaerobically. The conditions governing toxin production are not understood. The organisms may show good growth but yield very little toxin. It is possible that such functions may be dependent on oxidation-reduction conditions in the medium, and this communication deals with observations of electrode potentials developed in cultures of *C. diphtheriae* under different conditions, with a view to possible correlation with the biological functions of the organisms.

Methods.

The apparatus and methods used were the same as those previously described in the study of haemolytic streptococci [Hewitt, 1930].

8 cc. of culture medium were introduced into each cell, the inoculum was a small loopful of the surface pellicle of a 24-hour broth culture of Park-Williams 8 strain of *C. diphtheriae* and the incubator temperature was 37°.

Results.

Peptone infusion broth (Fig. 1). Horse-flesh infusion broth containing 2 % of Difco peptone was used. The potential fell to $E_h = -0{\cdot}14$ v. during the first 10 hours' incubation and then more slowly, reaching a minimum value of just below $-0{\cdot}20$ v. in 75 hours. Even after 140 hours' incubation the potential remained below $-0{\cdot}15$ v. The potential-time curve obtained may be contrasted with that of an aerobic culture in the same broth of haemolytic streptococci. With the latter organisms the potential falls rapidly to a minimum value of $-0{\cdot}17$ v. and then rapidly rises.

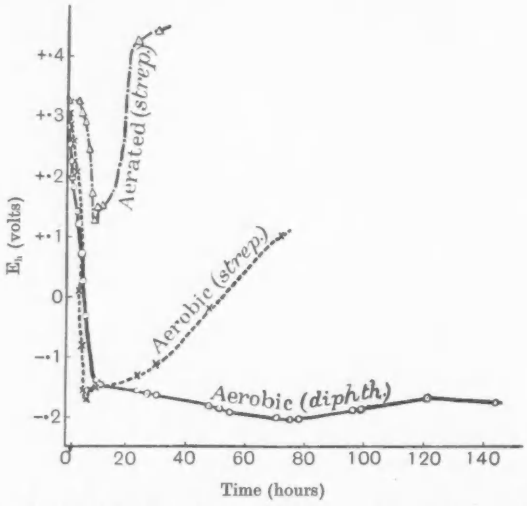

Fig. 1. Peptone infusion broth. *C. diphtheriae* and haemolytic streptococci.

Freshly heated broth (Fig. 2). Peptone infusion broth in tubes was immersed in a boiling water-bath for 30 minutes and cooled immediately before use. In the case of haemolytic streptococci the potentials of cultures in freshly heated broth fell twice as quickly and to a lower level than in untreated broth [Hewitt, 1930]. No appreciable difference, however, was observed between the potential-time curves of cultures of *C. diphtheriae* in heated and unheated broth.

Tryptic digest broth (Fig. 2). *C. diphtheriae* frequently requires acclimatisation to fresh cultural conditions. When, therefore, an inoculum was made from peptone infusion broth cultures to tryptic digest broth, growth was very slow and poor and, in conformity with this, the potential fell extremely slowly. Even after 96 hours' incubation the potential had barely reached the value $E_h = -0{\cdot}05$ v.

Sealed cultures (Fig. 3). In cultures sealed with vaselin to exclude air the potential fell extremely slowly and did not attain the low level reached in

aerobic cultures. This indicates the necessity of the presence of oxygen to enable the organisms to develop intense reducing conditions. In the case of haemolytic streptococci also, access to air was found necessary for the rapid development of intense reducing conditions.

Aerated cultures (Fig. 3). When broth cultures of *C. diphtheriae* were aerated vigorously and continuously in a three-limbed cell by means of Ridley's [1928] mixing apparatus, growth was more luxuriant than in stationary cultures and was distributed throughout the medium and not confined to a surface pellicle. The potential fell to within 50 mv. of the minimum value reached in stationary cultures and then rose fairly slowly towards the level of the original broth. These results are entirely different from those obtained with haemolytic streptococci (indicated in Fig. 1).

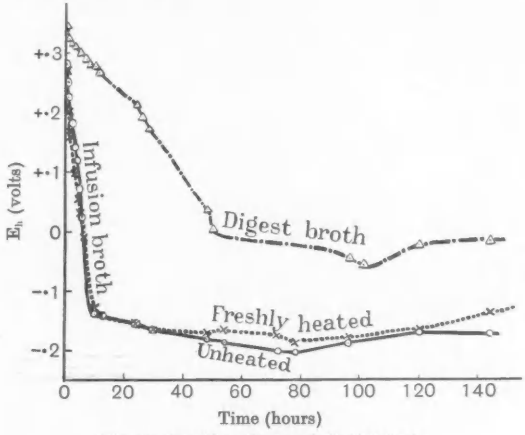

Fig. 2. Aerobic cultures of *C. diphtheriae*.

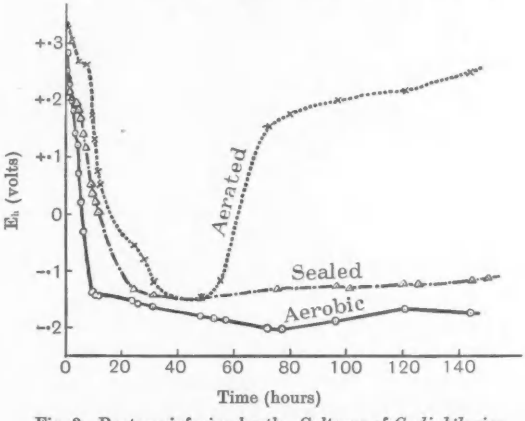

Fig. 3. Peptone infusion broth. Cultures of *C. diphtheriae*.

Table I. *Electrode potential changes in peptone infusion broth.*

Organism	Culture	Maximum fall in potential (mv.)
Streptococci	Stationary	480
,,	Aerated	200
C. diphtheriae	Stationary	500
,,	Aerated	480

With streptococci the fall in potential of aerated cultures was some 40 % of that in stationary aerobic cultures, whilst with *C. diphtheriae* the fall in aerated cultures was fully 90 % of that in stationary aerobic cultures.

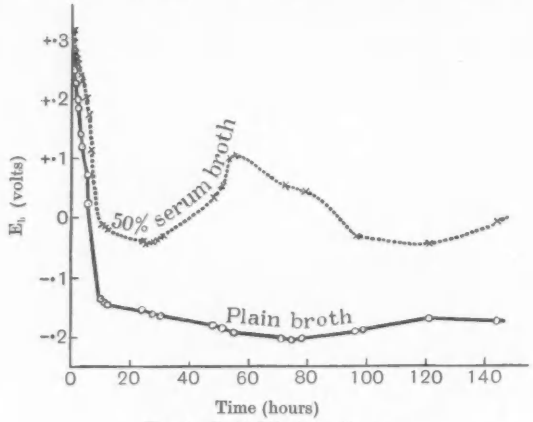

Fig. 4. *C. diphtheriae* cultures.

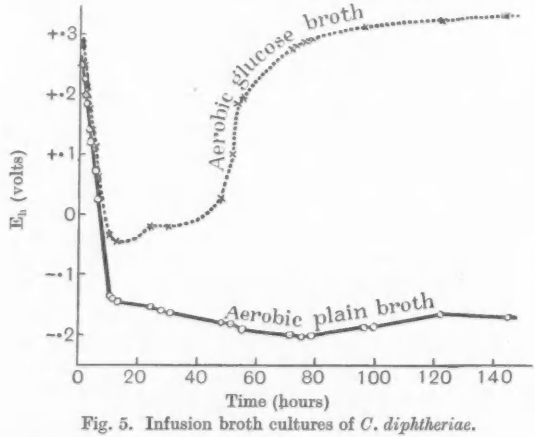

Fig. 5. Infusion broth cultures of *C. diphtheriae*.

After the phase of active growth the potential of streptococcal cultures rises rapidly to a high level, whilst the rise in potential of diphtheria cultures is relatively slow and a high (oxidising) potential is not reached.

Serum broth (Fig. 4). In 50 % serum broth the potential fell much less than in plain broth, as was observed with haemolytic streptococci.

Growth of *C. diphtheriae* appeared to cease much sooner in the presence of serum, but there is some evidence of a second fall in potential after some 60 hours' incubation.

Glucose broth (Fig. 5). For the first two days of incubation active proliferation occurred in 1 % glucose broth, but then ceased owing to accumulation of acid products. The potential did not fall to as low a level as in plain broth. This again is in conformity with results obtained with haemolytic streptococci.

Succinate broth (Fig. 6). Cultures of *C. diphtheriae* in broth containing 1 % of sodium succinate developed a deep yellow-brown colour and became very alkaline owing to oxidation of the succinate to carbonate. The surface pellicle assumed a corrugated shrunken appearance. The level of potential reached was not quite as low as in plain broth cultures and the potential began to rise after 4 days' incubation, possibly owing to the effect of the alkaline reaction on the organisms. In aerated succinate cultures also the brown colour and alkaline reaction were noted, and the potential did not fall to as low a value as in aerated cultures in plain broth.

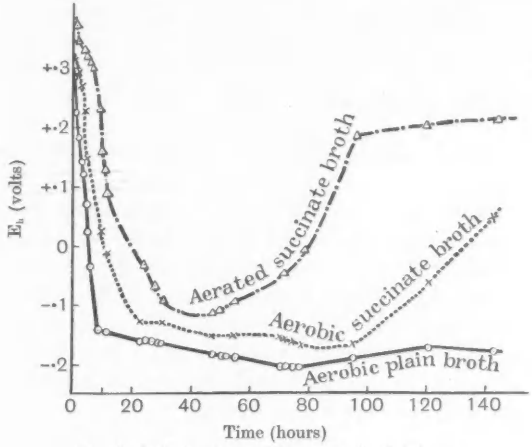

Fig. 6. Infusion broth cultures of *C. diphtheriae*.

Discussion.

In general the potential-time curves of cultures of *C. diphtheriae* are less susceptible to changes in cultural conditions than are those of cultures of haemolytic streptococci. This is probably due to the intense reducing ability of *C. diphtheriae*, which appears to multiply most readily when the oxygen supply is most abundant. As with haemolytic streptococci, the presence of oxygen appears to be necessary for the rapid development of intense reducing

conditions. In cultures sealed with vaselin to prevent access to air the potential falls very slowly. In aerated cultures, in which the oxygen supply is very abundant, growth is luxuriant and the potential falls to very nearly the same low level as in stationary aerobic cultures—the organisms being able easily to deal with the abundant oxygen supply and maintain reducing conditions in the medium until cessation of active proliferation.

The phase of active proliferation of *C. diphtheriae* is longer than that of streptococci and the lowest level of potential is reached more slowly, but the most marked distinction between the behaviour of the organisms is seen after the logarithmic phase of growth. With streptococci after 6–12 hours' incubation the potential begins to rise, but with *C. diphtheriae* no appreciable rise of potential is observed even after 140 hours' incubation. In aerated cultures the high level of potential corresponding to peroxide formation is rapidly attained in streptococcal cultures, but with *C. diphtheriae* this high level is not attained and the potentials do not exceed that of the original uninoculated broth. In the lack of oxidising function *C. diphtheriae* cultures are quite different from those of haemolytic streptococci, and this may be due entirely to the lack of ability to form peroxide.

The presence of serum prevents the potential of cultures of *C. diphtheriae* from falling to the low levels reached in broth. This oxygen-carrying effect, which was attributed tentatively to —S . S— groups in serum-proteins, is therefore not confined to cultures of haemolytic streptococci. Growth in serum broth was not luxuriant with *C. diphtheriae* as with haemolytic streptococci.

The effect of glucose on cultures of *C. diphtheriae* was similar to that on haemolytic streptococci, the acidity produced curtailing growth, but sodium succinate was utilised more readily by *C. diphtheriae* and on oxidation to carbonate produced an alkaline reaction which caused an early rise in potential.

The chief differences between the potentials of cultures of *C. diphtheriae* and haemolytic streptococci have been summarised in the following table. In place of the electrode potential, results are given in terms of the convenient numerical function r_H, which is the logarithm of the reciprocal of the partial pressure of hydrogen which is in equilibrium with the system. Oxidising systems have high r_H and reducing systems low r_H.

Table II. *Approximate values of* r_H *reached in cultures of* C. diphtheriae *and haemolytic streptococci.*

Culture medium	Organism	r_H after 6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Unboiled infusion broth ...	*C. diphtheriae*	15	9	8·5	8	7	8
" " ...	Streptococci	18	10·5	14	—	—	—
Freshly heated infusion broth	*C. diphtheriae*	15	9	8·5	8	7	8
" " "	Streptococci	9	9	9·5	13·5	18	—
Aerated infusion broth ...	*C. diphtheriae*	22·5	15·5	13	9	21	21
" " ...	Streptococci	20·5	18	30	30	—	—

SUMMARY.

1. More highly reducing conditions are developed in cultures of *C. diphtheriae* than with haemolytic streptococci, especially when the oxygen supply is most abundant.

2. After the logarithmic phase of growth the electrode potentials of cultures of *C. diphtheriae* do not rise rapidly, possibly owing to the lack of a peroxide-forming function.

3. The potential-time curves of cultures of *C. diphtheriae* are less susceptible to variation with cultural conditions than those of haemolytic streptococci, probably owing to the facts stated in the first two paragraphs.

4. Serum possesses an oxygen-carrying effect in cultures of *C. diphtheriae*.

The author is deeply indebted to Dr R. G. White and Dr E. W. Todd for their interest and encouragement.

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LXXVII. OXIDATION-REDUCTION POTENTIALS OF STAPHYLOCOCCAL CULTURES. I.

By LESLIE FRANK HEWITT.

From the Belmont Laboratories (L.C.C.), Sutton, Surrey.

(*Received May 2nd, 1930.*)

Results obtained with regard to the oxidation-reduction potentials of cultures of haemolytic streptococci [Hewitt, 1930, 1] and *C. diphtheriae* [Hewitt, 1930, 2] have been reported already. Close similarities in some respects and distinct differences in other points were traced in the behaviour of the two classes of organisms. In this communication the study is extended to *Staphylococcus aureus* cultures with the object of obtaining general rules concerning the oxidation-reduction potential changes occurring in bacterial cultures. Staphylococci, like the organisms previously studied, may be cultivated under a variety of aerobic and anaerobic conditions, but most luxuriant growth is observed when free access to oxygen is provided. The very wide range of values of oxygen tension over which many micro-organisms can multiply contrasts with the relatively narrow limits within which higher organisms can survive. This ease of adaptation to different respiratory conditions confers on bacteria the property of great variation in behaviour.

Methods.

The methods and apparatus employed have been precisely the same as those previously described [Hewitt, 1930, 1]. 8 cc. of medium were used in each experiment and the inoculum was 0·1 cc. of a 24-hour infusion broth culture of *Staph. aureus*.

Results.

Peptone infusion broth (Fig. 1). In ordinary stock horse-flesh infusion broth containing 2 % of Difco peptone the electrode potential fell to the level, $E_h = -0{\cdot}14$ v., within 9 hours. After this initial drop, the potential fell slightly during the next 140 hours, showing no tendency to rise. This behaviour is similar to that of cultures of *C. diphtheriae* in which the potential remains at a low level for a long period, but quite different from that of haemolytic streptococci. In the latter case the potential begins to rise immediately after the cessation of active proliferation. A distinct point of inflection on the potential-time curves is seen (Fig. 2) indicating reduction processes occurring in two stages.

Freshly heated broth (Fig. 2). Infusion broth was heated for 30 minutes in a boiling water-bath and cooled immediately before use. The potential fell much more rapidly and to a lower level than in unheated broth. This phenomenon was observed with haemolytic streptococci also, but not with *C. diphtheriae*.

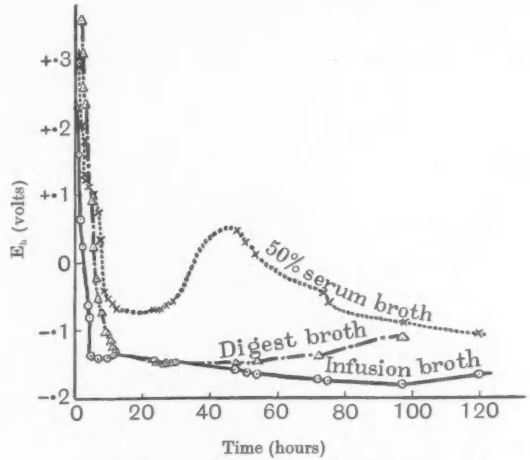

Fig. 1. Aerobic cultures of *Staph. aureus*.

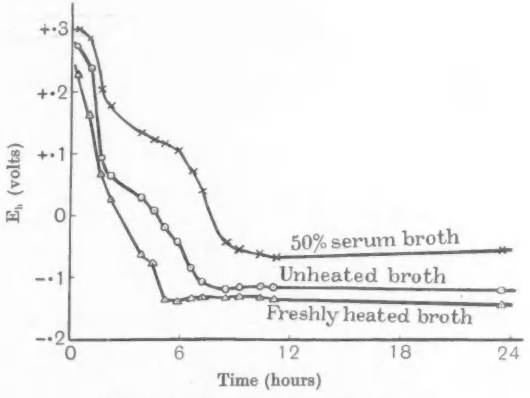

Fig. 2. Aerobic cultures of *Staph. aureus*.

Tryptic digest broth (Fig. 1). The potential fell quite rapidly in cultures in Douglas's tryptic digest medium. With haemolytic streptococci this sample of broth had a slight oxygen-carrying effect but it had very little with staphylococcal cultures.

Aerated cultures (Fig. 3). When cultures were aerated continuously and vigorously by means of Ridley's [1928] mixing apparatus growth was much more luxuriant than in stationary cultures. The potential fell rather more slowly and not to as low a level as in stationary aerobic cultures. The difference in potentials reached in aerated and aerobic cultures is much less than in the case of haemolytic streptococci but greater than with *C. diphtheriae*.

Table I. *Minimum electrode potentials reached in cultures.*

	E_h in volts		
	Streptococci	Staphylococci	*C diphtheriae*
Aerobic	− 0·16	− 0·155	− 0·20
Aerated	+ 0·12	− 0·055	− 0·15
Difference	0·28	0·10	0·05

The curve obtained with aerated cultures of haemolytic streptococci is given in Fig. 3 for comparison.

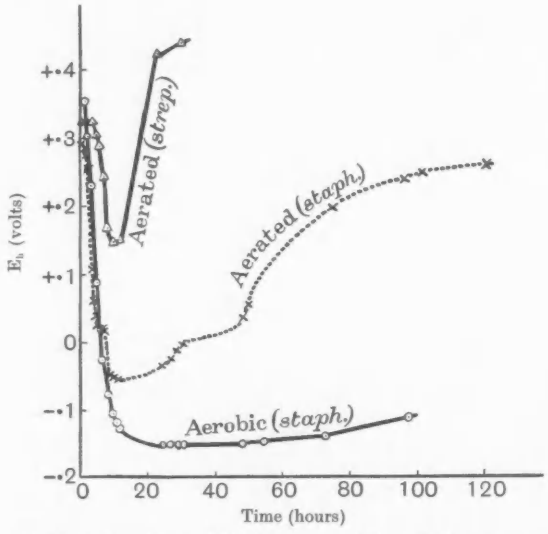

Fig. 3. Broth cultures of *Staph. aureus* and haemolytic streptococci.

Serum broth (Figs. 1 and 2). In 50 % serum broth cultures the potential did not fall to as low a level as in plain broth cultures. The form of the potential-time curves is similar to that obtained with haemolytic streptococci and possesses the same points of inflection. A further interesting observation is the second fall in potential in serum broth cultures after some 48 hours' growth. This may be due to breakdown of serum-proteins by proteolytic enzymes possessed by staphylococci.

Glucose broth (Fig. 4). The potential of aerobic cultures in 1 % glucose broth falls fairly rapidly but not to as low a level as in plain broth, and then rises, probably owing to the accumulation of acid in the culture. This phenomenon was observed also with haemolytic streptococci and *C. diphtheriae*.

In aerated glucose broth cultures the potential did not fall to as low a level as in the stationary glucose broth culture (minimum value $E_h = + 0{\cdot}01$ v. in place of $- 0{\cdot}08$ v.). After a period of increase in potential, however, probably associated with acid production, the potential fell for a second time and after 3–6 days' growth the potential in the aerated culture was actually lower than in the stationary aerobic culture, despite the oxidising effect of the increased oxygen supply in the aerated culture. It was found that the stationary culture had p_H 5·4, whilst in the aerated culture the p_H was 7·0 and growth was more luxuriant. It is evident, therefore, that the extra oxygen supply in the aerated culture had enabled the organisms to utilise the acids liberated during the glucose fermentation. The oxidation of lactate, etc., to carbonate accounts for the more alkaline reaction in the aerated culture.

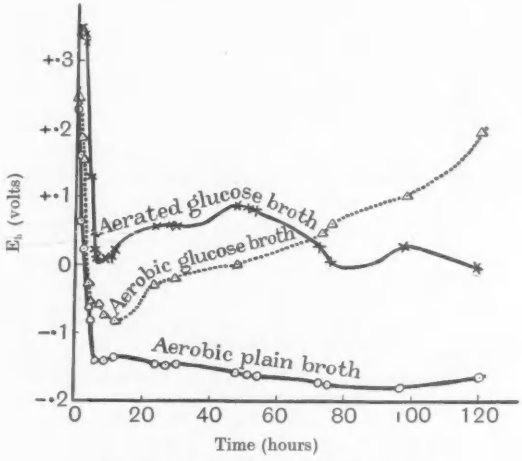

Fig. 4. Aerobic cultures of *Staph. aureus*.

Lactate-fumarate broth. In broth containing 1 % of sodium lactate and fumarate the potential fell to the same level as in plain broth but rose fairly rapidly. The reaction did not become alkaline and growth was relatively poor, indicating that the organisms were unable to utilise the acids in stationary aerobic cultures to any great extent.

Glycerol broth (Fig. 5). Good growth was observed in stationary aerobic 1 % glycerol broth cultures and the potential-time curve was practically indistinguishable from that of a plain broth culture. The culture did not become as acid as glucose broth cultures (p_H 6·6 in place of 5·4).

Thiodiglycollate broth (Fig. 5). In broth cultures containing 1 % of sodium thiodiglycollate the potential fell rather more slowly and did not reach the low level reached in plain broth cultures. There were also points of inflection on the curve similar to those obtained with haemolytic streptococci.

Discussion.

Staphylococci multiply most readily under conditions of abundant oxygen supply, and are vigorous reducing agents. In this they resemble *C. diphtheriae*; both organisms are more easily able to maintain intense reducing conditions in the presence of a generous oxygen supply than are haemolytic streptococci. In addition, neither *C. diphtheriae* nor staphylococci form peroxide: therefore the potential-time curves of cultures of these organisms remain flat at a low level of potential after the logarithmic phase of growth, whereas with streptococci the curve immediately begins to rise.

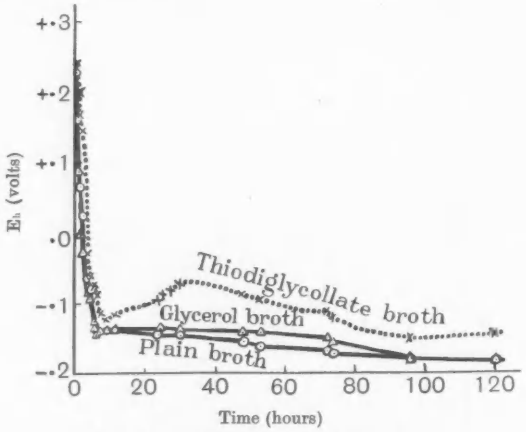

Fig. 5. Aerobic cultures of *Staph. aureus*.

Staphylococci resemble streptococci and differ from *C. diphtheriae* in the effect of freshly heated broth. In the case of the two former organisms the potential falls more rapidly and to a lower level in the freshly boiled broth. With *C. diphtheriae* no effect is seen.

With all three organisms the potential does not fall to as low a level in serum broth cultures as in plain broth. The oxygen-carrying effect of serum-proteins appears therefore to be a general one.

In glucose broth cultures also the three organisms are similar in that the potential does not fall to as low a level as in plain broth. With staphylococci, however, another effect is seen. Acids liberated during glucose fermentation are utilised much more readily in vigorously aerated cultures than in stationary aerobic cultures.

The presence of glycerol appears to have little effect on the potential-time curves of cultures, does not lead to acid production, and is therefore quite different in its effects from that of glucose.

When staphylococcal cultures are sealed autolysis proceeds and the culture clears. Little effect on the potential is observed, which suggests that the potential of the intracellular contents is very little different from that of the medium, under the conditions employed.

For comparison with previous results there are given in the following table the values of r_H (logarithm of reciprocal of partial pressure of hydrogen in equilibrium with the system) reached in *Staphylococcus aureus* cultures.

	Approximate r_H after					
Nature of culture	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	120 hrs.
Unheated broth culture	12	10	10	10	10	9·5
Freshly heated culture	9·7	9·7	9·3	9	8·5	8·0
Aerated broth culture	15	12	12·5	15	20·5	22

Summary.

1. *Staphylococcus aureus* resembles *C. diphtheriae* in maintaining more intense reducing conditions when the oxygen supply is abundant than haemolytic streptococci.

2. Probably owing to lack of peroxide-formation the potential in staphylococcal cultures remains at a low level for a long period, again like diphtheria and unlike streptococci.

3. In freshly heated broth the potential falls more rapidly in staphylococcal cultures, as with streptococci but not with *C. diphtheriae*.

4. Serum has an oxygen-carrying effect in cultures of staphylococci as with the other organisms investigated.

5. It is found with staphylococci, as with streptococci and *C. diphtheriae*, that the potential does not fall to as low a level in glucose broth as in plain broth; but with staphylococci an abundant oxygen supply facilitates the utilisation of acids liberated during glucose fermentation.

6. Glycerol, unlike glucose, has little effect on the potential-time curve.

The author wishes to acknowledge his indebtedness to Dr R. G. White and Dr E. W. Todd for their ready help, interest and encouragement.

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LXXVIII. THE FAT-SOLUBLE VITAMINS OF MILK.

By MARGARET EMILY FRANCES CRAWFORD, JOHN GOLDING, EDITH OLGA VERONICA PERRY and SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London, and the National Institute for Research in Dairying, Reading.

Report to the Medical Research Council.

(*Received May 6th, 1930.*)

Some years ago McCollum, Simmonds and Steenbock [1917] made the observation that fat-free milk, when included in a diet consisting of purified food substances, promoted growth and prevented decline of animals and, further, that melted butter-fat lost its growth-promoting capacity after being thoroughly agitated with twenty successive portions of water. This led them to suspect that the "fat-soluble A factor" might be appreciably soluble in water. Later, Steenbock, Boutwell and Kent [1918] were able to demonstrate that the "fat-soluble vitamin" was not actually removed from the butter by the water but that it was destroyed in the process of washing. Drummond, Coward and Watson [1921] using a basal diet free from both fat-soluble vitamins (the separate identity of the fat-soluble vitamins was not appreciated at that time) came to the conclusion that butter was less potent as a source of "fat-soluble A factor" than the same amount of fat supplied in the original milk and that the loss incurred was partly mechanical and partly due to destruction. As far as we are aware no strictly quantitative experiments have since been carried out to demonstrate whether the growth with the "fat-free" milk observed by McCollum, Simmonds and Steenbock was due to the presence of the fat-soluble vitamins present in the residual fat of the skim milk, which we presume their milk contained, or whether one or both of the fat-soluble vitamins of milk-fat is really appreciably soluble in water. The nature of an enquiry in which one of us (S.S.Z.) is at present engaged necessitated that this should be satisfactorily established and the experiments described in this communication were undertaken with this end in view.

Methods.

The morning milk from a typical herd of nine short-horn cows, which are kept for the study of normal milk at the National Institute for Research in Dairying, Shinfield, Berks., was used in this experiment. The sample was collected on May 13th, 1929. The cows were on pasture day and night and in addition received a mixture consisting of dried grains two parts and yellow maize germ meal one part.

This concentrate mixture was given to six cows which were in full milk, in quantities which averaged 6·5 lb. per day per cow, varying from 0 to 12 lb. according to the milk yield.

The milk contained 3 % fat and 12·05 % total solids. 25·75 lb. of the whole milk was dried in a vacuum pan under diminished pressure (75·75 cm.) for 5 hours; the temperature of the milk never rose above 36°. When the milk had reached a suitable concentration, the drying was continued in an evacuated desiccator over P_2O_5 at 37° until the water content was 22·15 %. It was then transported to the Lister Institute, London, and kept there in the cold room during the entire testing period. Likewise the separated milk from 36·1 lb. of the original milk, containing 0·1 % of fat was concentrated to a water content of 32·75 %. The separated cream was churned and the butter-milk thus obtained concentrated. The latter concentrate was added in equivalent quantities to the dried separated milk during the tests. The butter contained 11·7 % water. The dried milks were acid and had an odour of butyric acid.

In the butter, vitamin A was determined as previously described [Golding, Soames and Zilva, 1926] by the modified Steenbock method, daily doses of 0·1 g., 0·2 g. and 0·6 g. being employed. The estimation of vitamin A in the whole and in the skim milk plus butter-milk was carried out by administering daily the dose of dried milk mixed with an equal quantity of the basal diet [see Golding, Soames and Zilva, 1926] from which the caseinogen had been eliminated. After the animals had consumed the dose, the basal diet was offered *ad lib.* In the case of the whole milk, doses approximately equivalent to 0·05 g., 0·1 g. and 0·2 g. and in that of the skim milk plus butter-milk to 0·2 g., 0·5 g. and 0·7 g. of butter were tested. The animals apart from their doses consumed their basal diet willingly and in ample quantities (12–15 g. *per diem*).

Vitamin D in the whole and in the skim milk could not be assayed by a method in which McCollum's diet No. 3143 was used, because the daily consumption of the necessary quantities of dried milk equivalent to the butter doses would have also entailed the consumption of significant quantities of phosphorus, in which this diet is characteristically deficient. In fact, preliminary tests have actually shown that no satisfactory results could be obtained under the circumstances. Recourse was had to another method, namely that of Soames and Leigh-Clare [1928]. This method is based in the main on the increase in weight in young rats produced by the addition of vitamin D to a diet free from this vitamin but otherwise adequate. The basal diet employed in this research was Soames and Leigh-Clare's diet No. 2 and was made up as follows:

	Parts by weight
Wheat starch	43·7
Inactivated caseinogen ...	14·3
Cotton seed oil (hardened)	12·4
Yeast	8·0
Salt mixture	5·0
Lemon juice	5·0
Wheat germ	20·0
Water	50·0

The daily doses of dried milk were given in equivalent quantities of the following mixture:

	Parts by weight
Starch	79
Hardened cotton seed oil ...	21
Yeast	14
Salt mixture	9
Lemon juice	9
Wheat germ	36
The dried whole or skim milk	125
Water	82

After the consumption of the test dose, the above basal diet was offered *ad lib.* and quantities adequate to induce good growth in presence of sufficient vitamin D were consumed by the animals. In addition to the growth index, the ratio (A/R ratio) of the amount of mineral ash to the amount of organic material contained in the fat-extracted bone [see Chick, Korenchevsky and Roscoe, 1926] was also determined as suggested by Soames and Leigh-Clare.

Discussion of results.

Vitamin A.

The skim milk to which, as already mentioned, the butter-milk from the churning of the butter was added, contained of course a certain amount of the milk-fat. Thus the highest dose of 2·8 g. which was equivalent to 0·68 g. of butter contained 0·066 g. of fat, the second dose of 2·1 g. which was equivalent to 0·51 g. of butter contained 0·050 g. of fat, and the lowest dose of 0·7 g. which was equivalent to 0·17 g. of butter contained 0·016 g. of fat. Were any appreciable quantity of vitamin A associated in the milk after separation or in the butter-milk after churning with constituents other than fat, or should the vitamin have been more concentrated in the residual fat than in the butter, it would have been reflected in the growth curves of the rats receiving the "skim milk." By examining the growth curves of these animals, however (Fig. 1), it is seen that this was not the case, but that the growth obtained was more or less proportional to the residual fat present in the doses. Thus the lowest dose containing 0·016 g. of residual fat induced no appreciable growth, but the higher doses gave much better growth. This is particularly marked in the case of the highest dose containing 0·066 g. of residual fat. In fact the growth obtained with this dose was almost as good as that obtained with 0·1 g. of the butter (group VI, Fig. 2), which suggests that the minimum dose of milk-fat lies between these quantities. The weight curves of the rats receiving the whole milk (Fig. 2) also indicate that the vitamin A content of the doses was proportional to their milk-fat content, since the daily minimum amount of milk-fat necessary to induce optimum growth lies in this case between 0·05 g. (group III, Fig. 2) and 0·10 g. (group II, Fig. 2), a minimum also indicated by the growth curves obtained with the "skim milk" and with the butter.

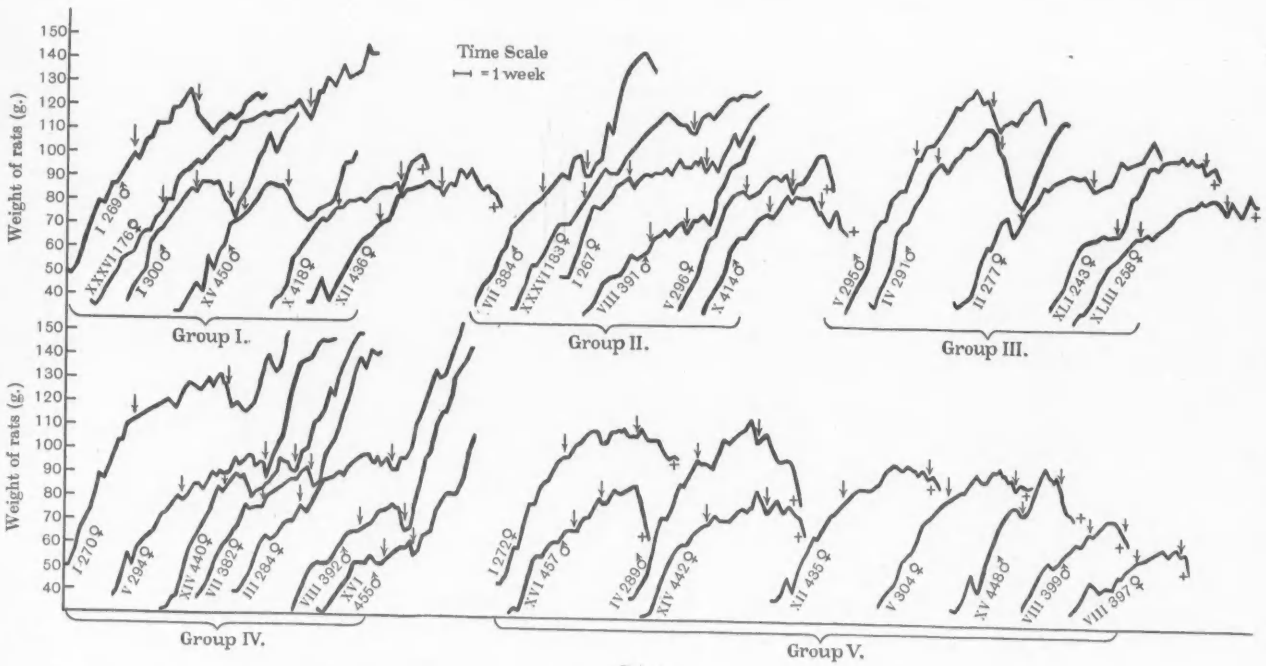

Fig. 1.

Group. I. Daily dose 2·80 g. of dried skim milk and butter-milk, equivalent to 0·68 g. of butter and containing 0·066 g. of milk-fat.
Group II. Daily dose 2·10 g. of dried skim milk and butter-milk, equivalent to 0·51 g. of butter and containing 0·050 g. of milk-fat.
Group III. Daily dose 0·70 g. of dried skim milk and butter-milk, equivalent to 0·17 g. of butter and containing 0·016 g. of milk-fat.
Group IV. Positive controls; 5 drops of cod-liver oil daily.
Group V. Negative controls.

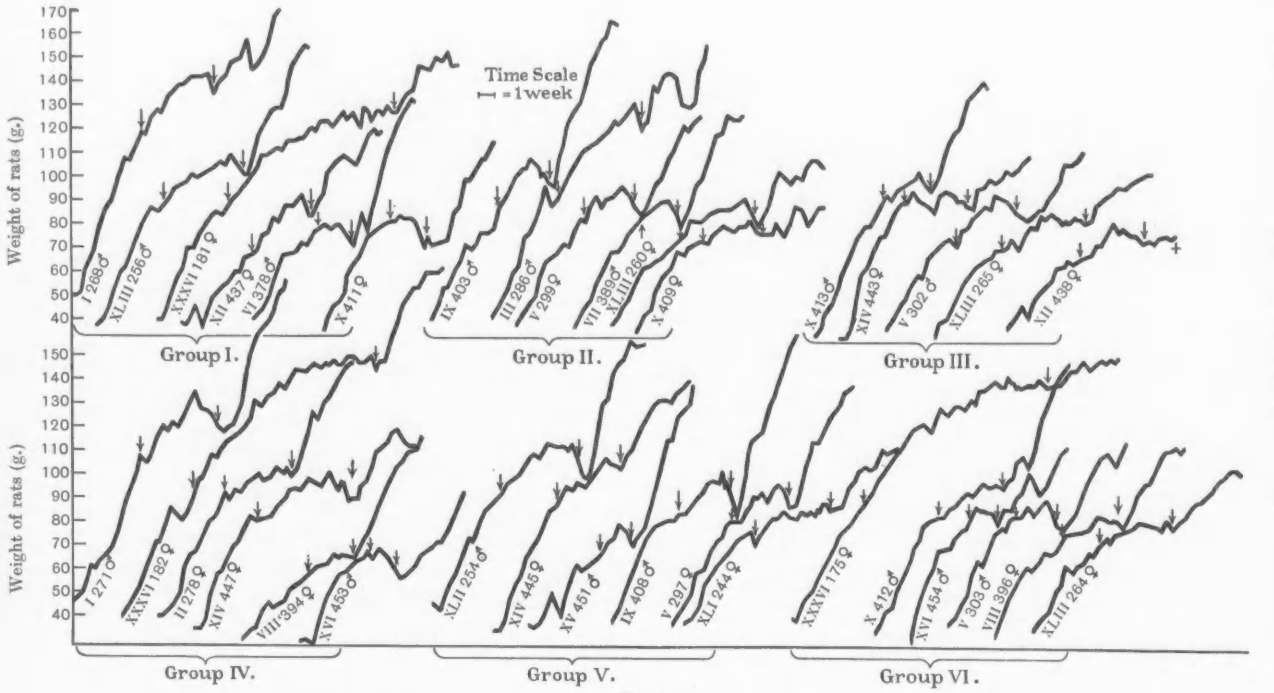

Fig. 2.

Group I. Daily dose 1·0 g. of dried whole milk, equivalent to 0·20 g. of butter and containing 0·21 g. of milk-fat.
Group II. Daily dose 0·50 g. of dried whole milk, equivalent to 0·10 g. of butter and containing 0·10 g. of milk-fat.
Group III. Daily dose 0·25 g. of dried whole milk, equivalent to 0·05 g. of butter and containing 0·05 g. of milk-fat.
Group IV. Daily dose 0·6 g. of butter. Group V. Daily dose 0·2 g. of butter. Group VI. Daily dose 0·1 g .of butter.
First arrow indicates beginning of the administration of irradiated cotton seed oil (vitamin D), second arrow that of the test dose.
+ denotes death.

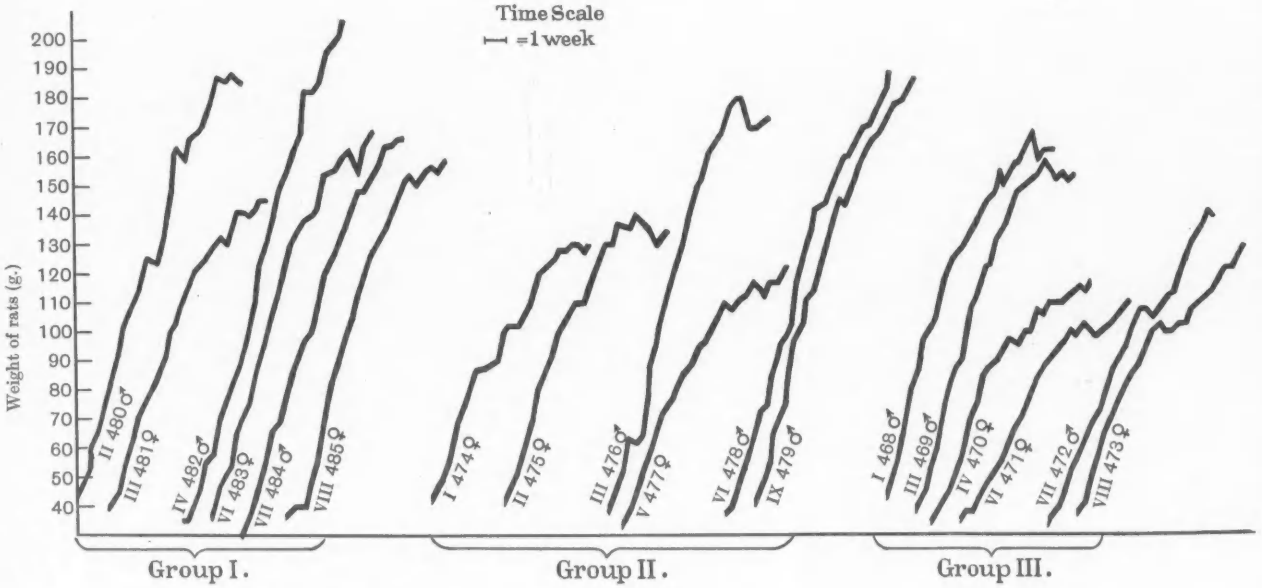

Fig. 3.

Group I. Daily dose 1·0 g. of dried whole milk, equivalent to 0·20 g. of butter and containing 0·21 g. of milk-fat.
Group II. Daily dose 0·50 g. of dried whole milk, equivalent to 0·10 g. of butter and containing 0·10 g. of milk-fat.
Group III. Daily dose 0·25 g. of dried whole milk, equivalent to 0·05 g. of butter and containing 0·05 g. of milk-fat.

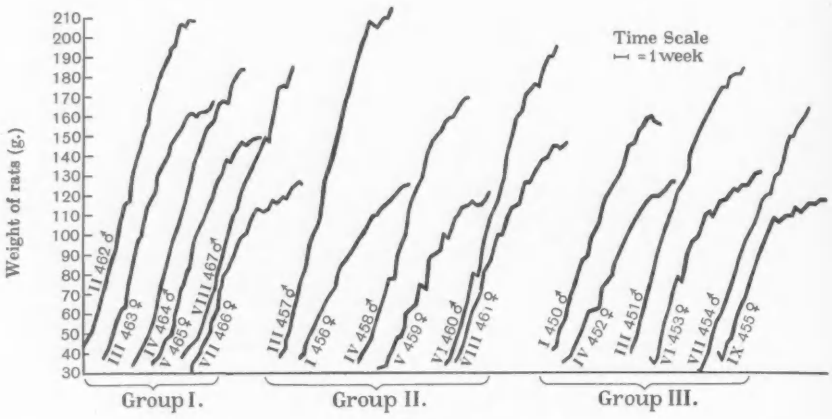

Fig. 4.

Group I. Daily dose 0·6 g. of butter.
Group II. Daily dose 0·2 g. of butter.
Group III. Daily dose 0·1 g. of butter.

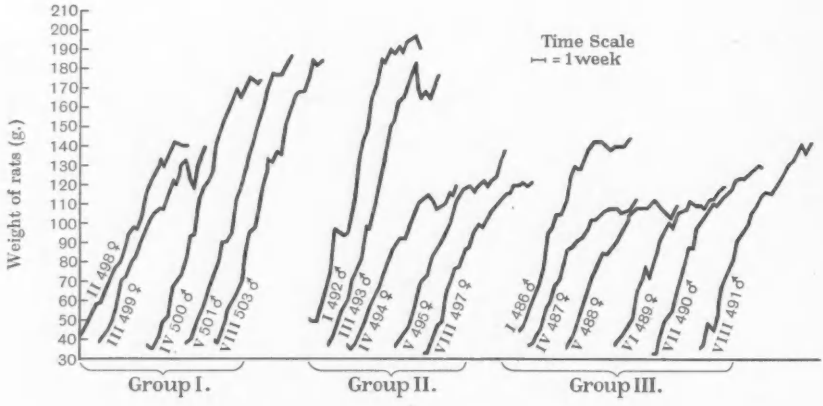

Fig. 5.

Group I. Daily dose 2·80 g. of dried skim milk and butter-milk, equivalent to 0·68 g. of butter and containing 0·066 g. of milk-fat.
Group II. Daily dose 2·10 g. of dried skim milk and butter-milk, equivalent to 0·51 g. of butter and containing 0·050 g. of milk-fat.
Group III. Daily dose 0·70 g. of dried skim milk and butter-milk, equivalent to 0·17 g. of butter and containing 0·016 g. of milk-fat.

Vitamin D.

Although the limitations of the method for the determination of vitamin D employed in this research have not yet been fully studied, we are of the opinion that the results obtained leave little doubt that, as in the case of vitamin A, vitamin D is associated entirely with the milk-fat. The growth-promoting capacity of the skim milk was again proportional to the amount of residual fat present in the dose. In these tests the minimum daily dose which induced optimum growth (as compared with the positive control group) was between 0·05 g. and 0·066 g. (groups II and I, Fig. 5), which was also more or less the

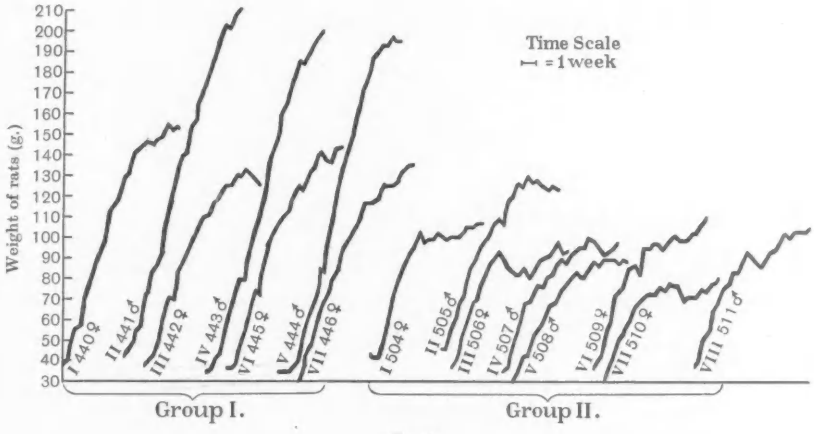

Fig. 6.

Group I. Positive controls; 5 drops of cod-liver oil daily.
Group II. Negative controls.

minimum dose indicated by the whole milk and butter tests (Figs. 3, 4 and 6). The analysis of bones of these rats (Table I and Fig. 7) showed that the A/R ratios were also more or less proportional to the amount of milk-fat present in the dose. These experiments showed that an optimum average A/R ratio was attained only when the rats received a daily dose of 0·2–0·6 g. of butter, a dose higher than that required to induce optimum growth. The animals on a dose of dried "skim milk" containing 0·066 g. of milk-fat (group IX, Fig. 7) showed a somewhat higher average A/R ratio than the animals receiving 0·1 g. of butter equivalent to 0·088 g. of fat (group I, Fig. 7)[1]. This, however, we consider as an error inherent to the method rather than an indication that the residual fat in the skim milk was more potent than the butter-fat.

[1] Also the animals on the dose 2·1 g. of dried skim milk containing 0·05 g. of milk-fat showed a slightly higher ratio than the animals receiving the dose 0·25 g. of whole dried milk containing 0·05 g. of fat.

Table I.

Negative controls

Rat	*A*/*R*
I 504 F.	1·18
II 505 M.	1·18
III 506 F.	1·20
IV 507 M.	1·06
V 508 M.	1·04
VI 509 F.	1·36
VII 510 F.	1·23
VIII 511 M.	1·17
Average	**1·18**

Positive controls

Dose	Rat	*A*/*R* Ratio
5 drops cod-liver oil daily	I 440 F.	1·59
	II 441 M.	1·45
	III 442 F.	1·55
	IV 443 M.	1·58
	V 444 M.	1·42
	VI 445 F.	1·59
	VII 446 F.	1·59
	VIII 447 M.	—
	Average	**1·54**

Butter

Dose	Rat	*A*/*R*
0·1 g.	I 450 M.	1·32
	III 451 M.	1·33
	IV 452 F.	1·54
	VI 453 F.	1·48
	VII 454 M.	1·39
	IX 455 F.	1·37
	Average	**1·40**
0·2 g.	I 456 F.	1·36
	III 457 M.	1·42
	IV 458 M.	1·48
	V 459 F.	1·46
	VI 460 M.	1·41
	VIII 461 F.	1·55
	Average	**1·45**
0·6 g.	II 462 M.	1·42
	III 463 F.	1·65
	IV 464 M.	1·47
	V 465 F.	1·06
	VII 466 F.	2·36
	VIII 467 M.	1·40
	Average	**1·56**

Dried whole milk

Dose	Rat	*A*/*R*
0·25 g. approx. equiv. to 0·05 g. butter and contained 0·05 g. of milk-fat	I 468 M.	1·28
	III 469 M.	1·28
	IV 470 F.	1·37
	VI 471 F.	1·36
	VII 472 M.	1·37
	VIII 473 F.	1·40
	Average	**1·34**
0·5 g. approx. equiv. to 0·1 g. butter and contained 0·1 g. of milk-fat	I 474 F.	1·33
	II 475 F.	1·44
	III 476 M.	1·40
	V 477 F.	1·37
	VI 478 M.	1·36
	IX 479 M.	1·38
	Average	**1·38**
1·0 g. approx. equiv. to 0·2 g. butter and contained 0·21 g. of milk-fat	II 480 M.	1·42
	III 481 F.	1·46
	IV 482 M.	1·42
	VI 483 F.	1·61
	VII 484 M.	1·40
	VIII 485 F.	1·59
	Average	**1·48**

Dried skim and butter-milk

Dose	Rat	*A*/*R*
0·7 g. approx. equiv. to 0·17 g. butter and contained 0·016 g. of milk-fat	I 486 M.	1·17
	IV 487 F.	1·34
	V 488 F.	1·26
	VI 489 F.	1·26
	VII 490 M.	1·41
	VIII 491 M.	1·29
	Average	**1·29**
2·1 g. approx. equiv. to 0·51 g. butter and contained 0·05 g. of milk-fat	I 492 M.	1·37
	III 493 M.	1·40
	IV 494 F.	1·43
	V 495 F.	1·47
	496	—
	VIII 497 F.	1·61
	Average	**1·46**
2·8 g. approx. equiv. to 0·68 g. butter and contained 0·066 g. of milk-fat	II 498 F.	1·46
	III 499 F.	1·56
	IV 500 M.	1·41
	V 501 M.	1·45
	502	—
	VIII 503 M.	1·53
	Average	**1·48**

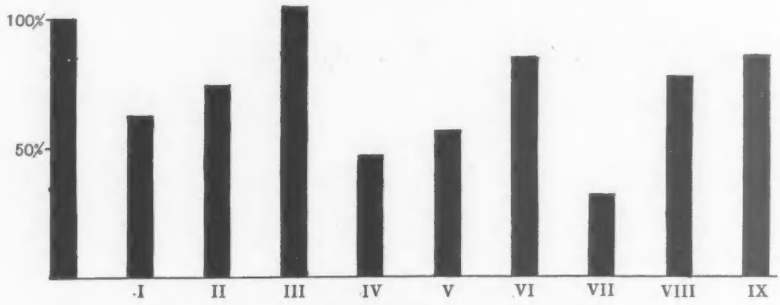

Fig. 7.

Percentage differences between average ratio of groups of animals and negative controls, ash/organic residue, not fat. (Difference between average ratio of positive group receiving daily 5 drops of cod-liver oil and negative group of each series = 100 %.)

Group I. Daily dose 0·1 g. of butter.

Group II. Daily dose 0·2 g. of butter.

Group III. Daily dose 0·6 g. of butter.

Group IV. Daily dose 0·25 g. of dried whole milk, equivalent to 0·05 g. of butter and containing 0·05 g. of milk-fat.

Group V. Daily dose 0·50 g. of dried whole milk, equivalent to 0·10 g. of butter and containing 0·10 g. of milk-fat.

Group VI. Daily dose 1·0 g. of dried whole milk, equivalent to 0·20 g. of butter and containing 0·21 g. of milk-fat.

Group VII. Daily dose 0·70 g. of dried skim milk and butter-milk, equivalent to 0·17 g. of butter and containing 0·016 g. of milk-fat.

Group VIII. Daily dose 2·10 g. of dried skim milk and butter-milk, equivalent to 0·51 g. of butter and containing 0·050 g. of milk-fat.

Group IX. Daily dose 2·80 g. of dried skim milk and butter-milk, equivalent to 0·68 g. of butter and containing 0·066 g. of milk-fat.

Summary.

The results obtained in these experiments suggest definitely that the entire vitamin A and vitamin D content of milk is associated with the milk-fat. There are no indications that these vitamins are more concentrated in one fraction of the milk-fat than in another, nor that there is a significant loss in either vitamin due to separating or churning, and consequently by testing the butters it is possible to obtain the approximate relative potency of milks. These experiments further suggest the utility of the Soames and Leigh-Clare method for the determination of vitamin D. The limitations of this method are being studied further.

We are indebted to Miss Dearden for the churning of the milk.

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LXXIX. VITAMIN A AND CAROTENE.

V. THE ABSENCE OF THE LIVER OIL VITAMIN A FROM CAROTENE.

VI. THE CONVERSION OF CAROTENE TO VITAMIN A *IN VIVO*.

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From the Nutritional Laboratory, Cambridge.

Report to the Medical Research Council.

(*Received May 3rd, 1930.*)

V. The absence of the liver oil vitamin A from carotene.

There is now general agreement that carotene of the highest purity so far attained possesses intense vitamin A activity [Euler, Euler and Hellström, 1928; Moore, 1929, 1, 2; Collison, Hume, Smedley-MacLean and Smith, 1929; Kawakami and Kimm, 1929; Hume and Smedley-MacLean, 1930], and the negative results of early workers must be attributed either to failure to supply vitamin D in the test diet, or more probably to insufficient precautions against oxidation of the pigment during the administration of the test doses.

Nevertheless it has been obvious for many years that carotene cannot be identified with the vitamin A of liver oils, since this factor must presumably be an almost colourless substance, differing notably from carotene in its solubility and adsorption properties. Thus vitamin A, present in unknown amounts in the pale orange-coloured liquid concentrates of cod-liver oils, is readily soluble in almost all organic solvents, including methyl alcohol and natural fats. Carotene, on the other hand, although not greatly exceeding the best cod-liver oil concentrates in physiological activity, is so deeply pigmented as to appear almost black in the pure state, and is readily soluble only in such solvents as chloroform, carbon disulphide and hot cyclohexane, being sparingly soluble in natural fats and almost insoluble in methyl alcohol. Similarly carotene is much more readily absorbed from its solutions by charcoal than vitamin A, the difference being so great as to afford an easy means of separation [Stephenson, 1920]. It is necessary, therefore, to account for the possession of a common physiological activity by two very divergent sources, and in the first place to ensure that the activity of carotene is not due to the presence of the liver oil factor in an adsorbed state, or in some other condition that would alter its normal solubility properties.

In supporting the contention that the activity of carotene samples might be due to contamination with the familiar form of vitamin A, Dulière, Morton

and Drummond [1929, 1] described a careful colorimetric and spectroscopic differentiation of carotene from the vitamin A of cod-liver oil. They found, in confirmation of Euler, Euler and Hellström, that the blue colours produced in the $SbCl_3$ reaction were of slightly different shade, that of carotene being characterised by an absorption band at 590 $\mu\mu$, that of vitamin A by a band at 608–612 $\mu\mu$, while in regard to the ultra-violet absorption spectra vitamin A was associated with a band at 320–330 $\mu\mu$, which was absent in the case of carotene. Carotene and the vitamin were therefore certainly different, but since it was also found that less pure specimens of carotene, similar to those found active by other workers, differed but little in their spectroscopic behaviour from highly purified samples, the additional suggestion was made that the presence of effective amounts of vitamin A in carotene might escape spectroscopic detection by reason of more intense superimposed absorption due to the pigment itself [Dulière, Morton and Drummond, 1929, 2].

Although Hume and Smedley-MacLean [1930] have now shown that even the most carefully purified carotene exhibits vitamin A activity, provided that the test doses are made up in a solvent in which decomposition does not take place, the above criticism would still seem to apply, since the persistence of traces of the liver oil vitamin A in carotene of the highest melting point might well be concealed if the absorptions due to the pigment were in fact of relatively overwhelming density. Capper [1930] has already shown that this condition does not hold good in regard to the absorption band at 328 $\mu\mu$. In the experiments described below it will similarly be shown that the blue colour given by carotene in the $SbCl_3$ reaction is of insufficient relative density to conceal the presence of effective amounts of the vitamin. This conclusion is in agreement with the previous qualitative observation that no sign of a maximum at 610–630 $\mu\mu$ could be observed in the colours produced in the $SbCl_3$ reaction by highly active samples of carotene [Moore, 1929, 1].

Experimental.

Method. Specimens of carotene (M.P. 174° and 178°) were compared colorimetrically with specimens of cod-liver oil concentrates (kindly supplied by Messrs Joseph Nathan and Co., Ltd., and Messrs Lever Brothers, Ltd.) using the technique previously described [Moore, 1929, 2]. The same materials were then tested biologically at doses graded to run parallel to the colorimetric values.

Colorimetric results. The chromogenic activities of the various materials were determined several times in each case and may be approximately represented by the following values[1].

[1] Although the tintometer technique certainly yields results of great value to the individual worker some uncertainty must be felt in expressing the values so obtained on an absolute scale. Apart from avoidable complications arising from variations in temperature and reagent [Wokes and Willimott, 1927; Evers, 1929, 1, 2] or from accelerated fading due to the presence of unsaturated acids [Norris and Danielson, 1929; Norris and Church, 1930] a personal element cannot be excluded in matching the shades produced. The results presented in the test, however, were obtained with the utmost care on the same occasion, and can in any case be regarded as giving satisfactory relative values.

	Blue units per mg. per "1 cm. cube" $SbCl_3$ reagent
Carotene (M.P. 174°)	180 at 590 $\mu\mu$
„ (M.P. 178°)	180 „
Concentrate A	180 at 610–630 $\mu\mu$
„ B	270 „ „

Biological tests. The biological work was carried out in several separate sections, rats nos. 1, 2, 3, 4 (albinos), nos. 5, 6, 7, 8, 9, 10, 11, 12 (albinos), nos. 13, 14, 15 (piebalds), and nos. 16 (piebald), 17, 18, 19 (albinos), being used in successive series of experiments. The usual curative technique was invariably employed, all the animals receiving the vitamin A-free diet previously employed, with the exception of rats nos. 13, 14, 15, which were kept under the care of Miss V. R. Leader at the Sir William Dunn Institute, where they received a slightly different basal diet (caseinogen 23 %, rice starch 40 %, cane sugar 17 %, palm kernel oil 15 %, salt mixture 5 %, + marmite extract 7·5 %).

Since all the materials had been found in earlier experiments to give positive results at 0·01 mg. per rat daily, attention was directed to dosages slightly below this level. The test doses were made up in arachis oil, one of the carotene samples (M.P. 174°) being dissolved by warming in the oil, the other (M.P. 178°) being added to the oil in hot cyclohexane, which was then removed under diminished pressure. During the experiment all the test solutions were kept in cold storage except when actually in use, and to ensure that no serious deterioration had taken place were tested by the $SbCl_3$ reagent at the conclusion of the experiment. The growth curves obtained are shown in Fig. 1.

Discussion.

In the above experiments carotene was found to be effective at levels down to 0·004 mg. per rat per day, while, with the exception of a single rat recovering on 0·002 mg., lower doses were found to be ineffective. The more active of the two concentrates tested was effective in curing a single rat at 0·0033 mg. but negative results were obtained in all other cases. It would thus appear that weight for weight the carotene samples were of slightly greater biological activity than the concentrate.

From the parallel colorimetric determinations 0·004 mg. of carotene may be considered equivalent to 0·7 Lovibond blue unit at 590 $\mu\mu$, while 0·0033 mg. of concentrate A corresponds to 0·9 blue unit at 610–630 $\mu\mu$. The colour value given by a minimal dose of the pigment therefore appears to be slightly smaller than that given by a minimal dose of concentrate. Since the possibility of the concealment of effective amounts of the liver oil vitamin A in carotene would imply that the colour value given by a minimal dose of carotene should be much greater than that given by a minimal dose of the concentrate this result affords strong evidence against this view. When in addition the work of Capper on the absence of the 328 $\mu\mu$ absorption band from carotene is taken into

account the only reasonable conclusion to be reached is that the activity of the pigment certainly cannot be attributed to direct contamination with vitamin A, or at least with the chromogen associated with that factor in liver oils.

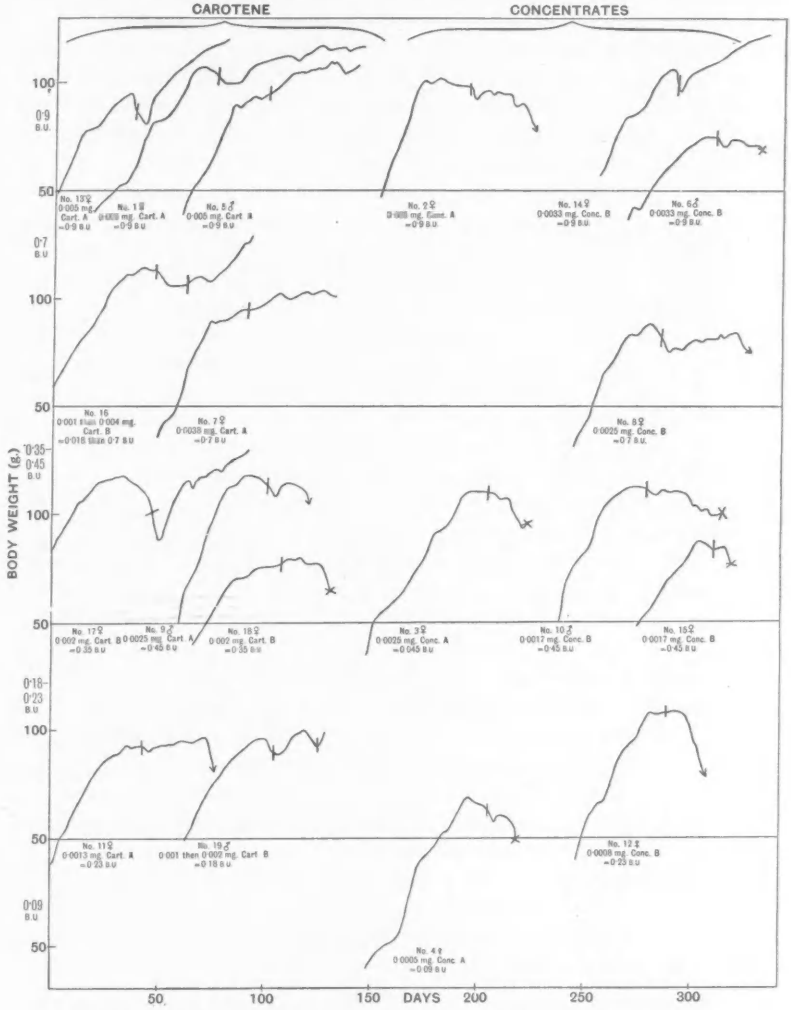

Fig. 1. The relative vitamin A potencies of carotene and cod-liver oil concentrates. B.U. = Lovibond blue ("1 cm. cube") unit. Cart. A = carotene (M.P. 174°). Cart. B = carotene (M.P. 173°). Conc. A, B = concentrate A, B.

VI. The conversion of carotene to vitamin A *in vivo*.

The question was once raised by Palmer [1919] as to whether it was logical to test the physiological activity of carotene in an animal, such as the rat, from which it was invariably absent. Answering the question in the negative Palmer and Kennedy [1920] attacked the question of the alleged identity of carotene with vitamin A by indirect means, and succeeded in proving conclusively that rats could thrive on diets almost completely freed from carotenoids. The assertion was therefore made that the pigment and vitamin were "neither identical nor necessarily associated."

The work of Euler, Euler and Hellström on the activity of carotene therefore seemed in conflict with preconceived ideas of the nature of the vitamin, while the later statement [Euler, Euler and Karrer, 1929, 1, 2] that excess of carotene could be stored in considerable amount in the liver oil of the rat was in direct contradiction to the finding of Palmer and Kennedy[1]. The Swedish workers, however, realised that carotene and the vitamin A of cod-liver oil were not identical, and suggested that several substances of carotenoid or "polyene" type might possess vitamin A activity.

In the first paper of this series [Moore, 1929, 1] it was pointed out that the "polyene" theory would imply an unexpectedly low order of specificity in vitamin A action, and the alternative suggestion was made that a chemical relationship between carotene and the vitamin might exist. Preliminary experiments supporting this view have already been described elsewhere [Moore, 1929, 3]. In the present paper more extensive evidence on the same point is presented.

Experimental method.

The method adopted in proving the conversion of carotene to the vitamin was essentially as follows. Young albino rats were "run out" upon a basal diet deficient in vitamin A [Moore, 1929, 1] until the "plateau" condition indicative of exhaustion of reserves of the vitamin was attained. Certain of the rats were then killed in this condition. To the remaining rats, varying amounts of carotene, either in the purified state or as red palm oil or carrots, were administered until complete cures and considerable increases in body weight had been effected. These rats were then killed.

The livers of all the rats were dissected out, finely minced, and mixed with twice their bulk of 5 % aqueous KOH, after which they were placed in cold storage until a suitable opportunity for examination arose. Even pro-

[1] (*Note added in proof.*) In a recent communication accessible to the present author only after the above paper went to press, Euler and Rydbom [*Svensk. Kemisk. Tidskrift*, 1929, **41**, 223] have shown that when rabbits are fed carrots or carrot extracts a "pale yellow coloured carotenoid," and not carotene, is stored in the liver. Although no attempt has been made to identify this "carotenoid" with the classical vitamin A, it is obvious that this result is in complete agreement with the experimental findings described in the present paper in the case of the rat.

onged storage did not lead to deterioration, although in most cases examinations were carried out in the course of a few days. On removal from cold storage the gelatinous masses were gently warmed until almost complete solution of the liver tissues was effected. The solutions were then extracted four times with about twice their volume of ether. The combined extracts in each case were washed thoroughly three times with water, and if necessary dried by shaking with anhydrous Na_2SO_4. The ether was then removed by evaporation under diminished pressure in a current of nitrogen, and the residual liver oils tested immediately for carotene and vitamin A by colorimetric methods.

The colorimetric differentiation of carotene from vitamin A.

It should be noted that, since carotene and the liver oil vitamin A both give positive results when tested biologically, it is unavoidable that resource to colorimetric data should be made in studying the conversion of one to the other. The validity of the colour reaction of the vitamin has indeed been questioned from time to time by several workers, but Drummond and his colleagues [see Ahmad and Drummond, 1930] have generally shown that such criticisms have been quite unjustified.

Suppose we compare the natural yellow colour of carotene itself in chloroform solution with the blue colours which it produces when it is dissolved in the same volume of $SbCl_3$ reagent. A yellow to blue ratio of about 11 to 1 is then found. (The yellow colour of the sample tested is replaced on treatment with the $SbCl_3$ reagent by a much less intense blue colour.) On the other hand, when we compare the faintly yellow colours of cod-liver oil concentrates with the corresponding $SbCl_3$ blue colours a ratio yellow to blue of about 1 to 100 is found. (The concentrate when so dilute as to be almost colourless will produce on treatment with the $SbCl_3$ reagent an intense blue colour.) Thus when one constituent is present in a given material in predominant amount the yellow to blue ratio suggests at once whether this constituent is a carotenoid or the almost colourless vitamin, while in the case of equal mixtures a rough working approximation as to the probable proportion is afforded.

In the present work the yellow to blue ratio was used as a first indication of the nature of the chromogen present in the liver oils. Results were then confirmed by the position of the absorption band produced in the $SbCl_3$ reaction (carotene 590 $\mu\mu$; vitamin A 610–630 $\mu\mu$). Certain samples were then despatched to Mr N. S. Capper for examination in regard to the ultra-violet absorption, while others were employed in biological tests.

Results.

The results obtained are shown in Table I. It will be seen that the liver oils of rats nos. 1 to 10, which had either died or been killed in a condition of acute avitaminosis A, invariably gave negative results when tested with the $SbCl_3$ reagent. The liver oils of rats nos. 11 to 14, in which growth had been restored by small doses of carotene, similarly gave negative results, as indeed would be

expected at levels sufficient to meet immediate needs but insufficiently liberal to permit storage of the vitamin. On the other hand, in the case of the liver oils of rats nos. 15 to 22, which received either purified carotene, red palm oil, or fresh carrots in considerable excess of the minimal dosage, strongly positive reactions were given, which in all cases could be attributed to the appearance of large amounts of vitamin A, and not to the storage of unchanged carotene.

Since the strongest evidence in favour of the conversion must be derived from those cases in which carotene of the highest purity was used, special attention may be first directed to rats nos. 15 to 18 which received carotene (M.P. 178° uncor.) which had been obtained from carrots and recrystallised twelve times from cyclohexane, operations being conducted for the most part in an atmosphere of nitrogen. The pigment was added in cyclohexane to the arachis oil used in compounding the basal diet, the cyclohexane being removed under diminished pressure. After having attained the "plateau" condition each rat received the carotene during from 16 to 26 days at the rate of 0·75 mg. per 10 g. of diet eaten, which was the average amount consumed daily. At autopsy, despite very liberal amounts of pigment ingested, the body fat was always found to be quite colourless, apart from occasional small loci in regions where the intra-peritoneal fat lay in contact with the gut wall. The liver oils were certainly yellower than the liver oils of the control rats which had received no carotene, but these increases in yellow values were trivial when contrasted with the enormous concentration of yellow pigmentation that would have been expected if the intense blue colours now given in the $SbCl_3$ reaction had been due to unchanged carotene. The following observations confirm the view that conversion had been effected. (1) These blue colours were now characterised in all cases by a well-defined band at 610–630 $\mu\mu$, the position characteristic of vitamin A, and not at 590 $\mu\mu$, the position characteristic of the ingested carotene. (2) In preliminary work by Mr N. S. Capper (private communication) it was found that the untreated liver oil now displayed the absorption band at 320–330 $\mu\mu$ characteristic of vitamin A. (3) As a final precaution against the possibility that the chromogen should represent an inactive degeneration product of the pigment the vitamin A activity of specimen liver oils was confirmed by biological tests (Fig. 2).

In regard to rats nos. 18 to 22 which received even more liberal supplies of carotene as red palm oil or carrots, similar results were obtained, in which the yellow and blue values of the liver oils attained somewhat higher levels. For example the liver of rat no. 21, which had received during several weeks a diet consisting largely of carrots, was bright orange in colour. Such obvious pigmentation might at first sight be taken to support the claim of Euler, Euler and Karrer that the carotene had been stored in an unchanged condition, but it may be pointed out that the blue value of the liver oil would have corresponded to the presence of about 80 mg. of the pigment, *i.e.* the liver oil would have appeared deep crimson and would have deposited crystalline carotene freely on standing.

Expression of results.

The colorimetric examination of the liver oils was carried out according to the technique previously described [Moore, 1929, 2]. The method of expressing results may be explained most simply by an actual example.

Rat no. 18. Liver oil dissolved in 10 cc. chloroform.

Yellow units. The colour of this solution was matched by Lovibond glasses 4 yellow, 0·7 red. Therefore total number of yellow units in liver = 4×10 (volume of solution in cc.) = 40 units.

Blue units. 0·05 cc. of the above solution was made up with washings of chloroform to 0·5 cc. $SbCl_3$ reagent (2 cc.) was then added. The resulting blue colour was matched by Lovibond glasses 4·0 blue, 1·0 yellow (average of several readings). Therefore total number of blue units

$$= 4{\cdot}0 \times \frac{2{\cdot}5 \text{ (volume of reaction mixture)}}{0{\cdot}05 \text{ (volume of solution used in test)}} \times 10 \text{ (volume of the original solution)}$$

= 2000 units.

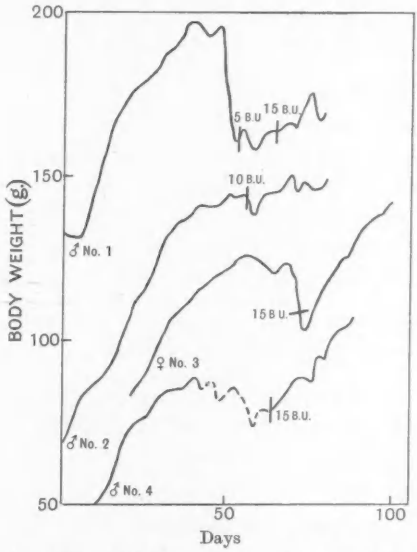

Fig. 2. The vitamin A activity of the liver oils of rats (no. 15 and 18 of Fig. 1) which had received excess of carotene.

Doses are expressed in Lovibond blue ("1 cm. cube") units. Rat no. 3 was completely cured of severe xerophthalmia and a septic cheek. N.B. Rat no. 4 received an ineffective material in a separate experiment during the period indicated by the broken curve.

It should be noted that the yellow and blue values so expressed are directly comparable. The method of calculation will be seen to be exactly the same in each case when it is remembered that the addition of the $SbCl_3$ reagent in obtaining the blue value necessarily involves a further dilution of the chromogen.

Table I. *Analyses of liver oils for vitamin A and carotene.*

Rat no. and sex	Body wts.: initial; plateau; final (g.)	No. of days on vitamin A-free diet	Dosage of carotene (mg.)	No. of days of carotene feeding	Total Lovibond units ingested by rat: Blue units in $SbCl_3$ reagent at 590$\mu\mu$	Total Lovibond units ingested by rat: Yellow units in $CHCl_3$	Total Lovibond units found in liver: Blue units in $SbCl_3$ reagent at 610–630$\mu\mu$	Total Lovibond units found in liver: Yellow units in $CHCl_3$	Total yellow units to be expected in liver if blue chromogen were carotene
	Rats receiving vitamin A-free diet. No treatment.								
1 ♂	54, 171, 124 (D)	77	—	—	—	—	0	4	—
2 ♂	41, 121, 105 (K)	67	—	—	—	—	0	9	—
3 ♂	45, 105, 89 (K)	67	—	—	—	—	0	5	—
4 ♀	42, 119, 109 (K)	51	—	—	—	—	0	6	—
5 ♂	54, 99, 92 (D)	33	—	—	—	—	0	2	—
6 ♀	31, 58, 58 (D)	26	—	—	—	—	0	2	—
7 ♀	33, 60, 45 (D)	39	—	—	—	—	0	1	—
8 ♀	85, 127, 105 (K)	54	—	—	—	—	0	5	—
9 ♂	140, 167, 145 (D)	50	—	—	—	—	0	10	—
10 ♀	62, 100, 77 (D)	54	—	—	—	—	0	3	—
	Rats cured by small doses of carotene (M.P. 174°).								
11 ♀	45, 111, 133 (K)	54	0·02	20	70	770	0	10	0
12 ♂	51, 128, 178 (K)	47	0·01	43	80	880	0	16	0
13 ♀	52, 114, 133 (K)	53	0·05	45	400	4,400	0	7	0
14 ♀	—, 89, 89 (K)	—	0·5	3	270	3,000	0	23	0
	Rats cured by large doses of carotene (M.P. 178°).								
15* ♂	38, 95, 159 (K)	41	0·75	16	2,200	24,000	2,000	90	22,000
16* ♀	33, 95, 139 (K)	41	0·75	22	3,000	33,000	3,300	110	36,000
17 ♀	36, 66, 106 (K)	29	0·75	26	3,500	39,000	3,700	100	41,000
18 ♂	41, 76, 137 (K)	28	0·75	26	3,500	39,000	2,000	40	22,000
	Rats cured by red palm oil.								
19 ♂	33, 73, 152 (K)	29	R.P.O. 1·5 g.	26	12,000	130,000	5,000	280	55,000
20* ♀	38, 94, 127 (K)	41	R.P.O. 1·5 g.	16	7,400	83,000	4,500	400	50,000
	Rats cured by fresh carrots.								
21 ♀	35, 86, 147 (K)	56	Carrots *ad lib.*	55	?	?	16,000	600	180,000
22 ♂	49, 114, 153 (K)	71	,,	35	?	?	5,300	250	58,000

D = Died. K = Killed.

* These rats were first cured by very minute doses of carotene before the larger amounts were given.

In calculating the colour value of the ingested pigment in Table I the values for both samples of carotene have been taken as blue = 180 units per mg. per cc., yellow = 2000 units per mg. per cc.

Discussion.

The above results afford a complete picture of the conversion of carotene *in vivo* to the familiar vitamin A of liver oils, as defined by all the chromogenic and spectroscopic properties which have been found to run parallel to this factor. The details of the conversion may be summarised as follows:

Carotene	⟶	Vitamin A
Synthesised in plant		Stored in animal
Intensely yellow		Almost colourless
328$\mu\mu$ absorption band absent		328$\mu\mu$ absorption band developed
Greenish blue $SbCl_3$ reaction at 590$\mu\mu$		Vivid blue $SbCl_3$ reaction at 610–630$\mu\mu$

This evidence could be regarded as conclusive if it could be stated with certainty that the carotene used was completely pure. Upon this point, however, doubts might be raised, since Euler, Karrer and Rydbom [1929] have

advanced certain evidence, based upon supposed variations in the activity of different samples of carotene, which raises the question as to whether the activity of the pigment may not after all be due to the presence of a "stubbornly adhering" impurity active at about the same level as vitamin D. The danger of this possibility was indeed pointed out by the writer [Moore, 1929, 4] at a time when Euler, Euler and Hellström were satisfied with the chemical purity of their carotene, but it is now obvious that this view in its simplest form would imply a complete rejection of the chromogenic evidence, since it is obvious that the same chromogen cannot be present in both carotene and cod-liver oil. It should be noted that the evidence of Euler, Karrer and Rydbom [1929] is based on small variations in the growth rates of rats and in blue values determined by the tintometer technique. Results obtained by both these methods are notably open to considerable experimental errors, and must therefore be of very doubtful significance.

A point of greater interest, however, arises from the statement of Drummond and Baker [1929] that vitamin A probably constitutes only a minute fraction (10 % and probably much less) of even the most potent liver oil concentrates. Experiments described above (Part V) have shown that the minimal dose of carotene is only slightly lower than that of the most active concentrates, which may actually exceed the pigment in regard to chromogenic activity. From this evidence it might be expected that the activity of carotene would similarly be derived from a small fraction of the total pigment. Two alternative possibilities, however, may be borne in mind, either that the conversion of carotene may be of an inefficient character, or that the estimate of Drummond and Baker may be unduly low. For the present the evidence does not seem of sufficient weight to justify any serious doubt that the physiological activity of carotene is not derived from the pigment *per se*.

Summary.

Part V.

1. The $SbCl_3$ colour reaction given by a minimal physiological dose of carotene at 590 $\mu\mu$ is slightly less than that given by a minimal physiological dose of the vitamin A of liver oil at 610–630 $\mu\mu$.

2. It is therefore impossible that the colour reaction of carotene could conceal any underlying colour reaction due to the liver oil vitamin A in such amounts as would account for its physiological activity.

Part VI.

1. The liver oils of rats suffering from vitamin A deficiency invariably gave negative results when tested with the $SbCl_3$ reagent.

2. Rats after depletion of vitamin A were cured by the administration of large excess of carotene (12 times recrystallised from cyclohexane, M.P. 178° uncor.), red palm oil, or fresh carrots. Traces of yellow pigment now appeared

in the liver oil but the predominant chromogen present (99 %) was invariably vitamin A, as characterised by (*a*) absence of such intense yellow pigmentation as must have accompanied the storage of carotene as such; (*b*) an intensely positive $SbCl_3$ reaction showing a marked band at 610–630 $\mu\mu$; (*c*) the development of an absorption band in the untreated oil at 328 $\mu\mu$; (*d*) intense biological activity.

3. The conclusion must therefore be reached that carotene, or some part thereof, if it should later prove to be heterogeneous, behaves *in vivo* as a precursor of the vitamin.

My thanks are due to Dr L. J. Harris for his valuable criticism, to Mr T. A. Webster for advice on the extraction of liver oils and to Messrs Chivers and Sons, Limited, for drying the large quantities of carrots from which the carotene used in this experiment was derived. The care of the experimental animals was in the capable hands of Mr A. Ward.

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LXXX. PHOSPHORIC ESTERS IN ALCOHOLIC FERMENTATION.

III. THE LAG BETWEEN PHOSPHATE ESTERIFICATION AND CARBON DIOXIDE EVOLUTION.

IV. OXIDATION-REDUCTION POTENTIALS OF YEAST PREPARATIONS.

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III. The lag between phosphate esterification and carbon dioxide evolution.

Euler and Johansson [1913] were the first to suggest that in the reaction of phosphate with yeast preparations fermenting sugar the phosphate was esterified some time before the equivalent carbon dioxide was liberated. These workers considered that a preliminary activation of sugar, followed by formation of hexosediphosphate and a triosemonophosphate, was responsible for the lag in evolution of gas. In addition to the lag between the reactions of phosphate esterification and carbon dioxide formation there is a delay in the esterification after the phosphate is added. When yeast preparations (particularly maceration extract and preparations containing much water) are added to sugar there is a considerable induction time before any fermentation occurs; the free phosphate present in the preparation then reacts during the period called the "Gäranstieg" by Meyerhof [1918]. This induction period and the "Gäranstieg" caused by the free phosphate inherent in preparations are not discussed in the present work.

In a recent paper on the reaction of phosphate with dried yeast fermenting fructose [Boyland, 1929] a lag similar to that of Euler and Johansson was described. This lag between the processes of esterification and carbon dioxide formation was not due to physical causes such as supersaturation, and it was suggested that it was due to the relatively slow decomposition of products formed after, or simultaneously with, phosphate esterification. The nature of this lag is investigated in the following experiments.

Harden and Young [1908] showed that in the reaction of phosphate with yeast preparations the time necessary for the attainment of the maximum rate of carbon dioxide evolution was much greater with glucose than with fructose,

and that this time could be reduced by the addition of relatively small quantities of fructose [Harden and Young, 1909]. Harden and Henley [1920, 1921] found that this time was much reduced by the addition of hydrogen acceptors such as methylene blue, pyruvates or aldehydes. Acetaldehyde was the most active agent in this way, being about fifty times as efficacious as fructose. Under almost all conditions the reaction of one molecule of phosphate in fermentation yields one molecule of carbon dioxide, whether monophosphoric or diphosphoric ester is formed. Combining the recent ideas of Harden [1930] with those of Neuberg and Kerb [1913], the sequence of fermentation in presence of a trace of aldehyde might be represented as follows:

(1) $3C_6H_{12}O_6 + 2R_2HPO_4 = 2C_6H_{11}O_5(PO_4R_2) + 2CH_3.CO.CHO + 4H_2O$,
(2) $2C_6H_{11}O_5(PO_4R_2) + C_6H_{12}O_6 + 2R_2HPO_4 = 2C_6H_{10}O_4(PO_4R_2)_2 + 2CH_3.CO.CHO + 4H_2O$,
(3) $CH_3.CO.CHO + CH_3.CHO = CH_3.CO.COOH + C_2H_5OH$,
(4) $CH_3.CO.COOH = CH_3.CHO + CO_2$.

Of these reactions the only one likely to be affected by hydrogen acceptors is the oxidation of methylglyoxal to pyruvic acid (equation 3), and as hydrogen acceptors have such a marked effect on carbon dioxide evolution this oxidation is probably the slowest or controlling stage in the beginning of the reaction of phosphate. It should therefore be responsible for the lag in the appearance of carbon dioxide after phosphate esterification and for this reason the effect of glucose, fructose and hydrogen acceptors on the lag was examined.

Experimental.

The preparation used in most of these experiments was the "zymase" free from phosphatase, made by the acetone precipitation of yeast juice, which has previously been described [Boyland, 1930]. It was possible to obtain reliable preparations free from hexosephosphatase by grinding the yeast juice precipitate with half its weight of kieselguhr together with the sugar and water necessary for the fermentation. The whole was then centrifuged and the clear centrifugate used for the fermentation. Harden and Macfarlane [1930] have indicated that hexosephosphatase is probably adsorbed by kieselguhr.

The methods used were largely the same as those of previous experiments, the analytical procedure being identical. When hydrogen acceptors were added, the addition was made 15 minutes before the addition of phosphate. The concentrations of the added substances are calculated on the final volume of the fermenting solution after the addition of phosphate.

Effect of hydrogen acceptors on the reaction.

The experiments of Table I, in which the lag is expressed as the difference between the phosphate esterified and the carbon dioxide evolved at any particular time, show that hydrogen acceptors cause a marked increase in the rate of reaction of phosphate, without diminishing the amount of lag as measured by molecular concentrations. Hydrogen acceptors sometimes increase the lag in amount, though not the period during which it persists; it may be possible

Table I. *The lag between phosphate esterification and carbon dioxide evolution in fermentation with the acetone precipitate of yeast juice.*

In each case 1 cc. 2*M* K_2HPO_4 added to 2 g. yeast preparation in 20 cc. sugar solution. Results in total millimols.

Time after phosphate addition (mins.)		5	10	20	30	40	50	60	70
Exp. 180									
Glucose alone	P esterified	0·08	0·20	—	0·83	—	1·65	2·00	2·00
	CO_2 evolved	—	0·09	—	0·63	—	1·55	2·10	2·10
	Lag	0·08	0·11	—	0·20	—	0·10	—	—
Exp. 203									
Fructose alone	P esterified	0·10	0·25	—	0·84	—	1·90	2·00	—
	CO_2 evolved	—	0·12	—	0·70	—	1·82	2·06	—
	Lag	0·10	0·13	—	0·14	—	0·08	—	—
Exp. 204									
Fructose with methylene blue (0·05 %)	P esterified	0·12	0·28	—	1·15	—	1·99	1·99	—
	CO_2 evolved	—	0·10	—	0·90	—	1·94	2·03	—
	Lag	0·12	0·18	—	0·25	—	0·05	—	—
Exp. 199									
Fructose with acetaldehyde (0·15 %)	P esterified	0·30	0·80	1·60	1·96	—	—	—	—
	CO_2 evolved	0·16	0·64	1·56	1·97	—	—	—	—
	Lag	0·14	0·16	0·10	—	—	—	—	—

Table II. *The effect of varying concentrations of acetaldehyde on the lag.*

10 cc. 0·6 *M* K_2HPO_4 added to 4 g. dried yeast in 30 cc. 10 % fructose. Concentrations expressed as molecules per litre.

Concentration acetaldehyde	Change after 20 minutes' reaction: P esterified	CO_2 evolved	Difference or lag
Nil	0·017	—	0·017
0·015	0·022	0·009	0·013
0·030	0·036	0·025	0·011
0·045	0·037	0·024	0·013
0·060	0·043	0·024	0·019
0·075	0·042	0·018	0·024

that in the presence of certain concentrations of acetaldehyde, pyruvic acid is formed more quickly than it can be decomposed by the carboxylase, and under such conditions the decarboxylation would become the master reaction. The lag occurs in the reaction with all the yeast preparations examined (acetone precipitate of yeast juice, yeast juice, zymin and dried yeast). It varies in extent with different preparations and must depend upon the balance of enzymes present. It is greater with larger additions of phosphate and it cannot be much reduced by any concentration of acetaldehyde. Table II shows the lag with dried yeast fermentations with varying concentrations of acetaldehyde. The results are also set out in Fig. 1 and the optimum concentration of acetaldehyde is fairly well marked.

As this lag is not removed by hydrogen acceptors—which increase both the rate of esterification and of carbon dioxide evolution—and is fairly constant in amount for any one preparation, it is probable that esterification is normally arrested by accumulation of methylglyoxal. The removal of methylglyoxal (by oxidation at the expense of hydrogen acceptors) allows more sugar to be esterified.

Triose in the reaction.

If the lag in fermentation is due to the relatively slow oxidation of methylglyoxal, then that compound should accumulate in fermentation when the lag is at its greatest. The triose present during the reaction was therefore estimated by the distillation method described by Neuberg, Färber, Levite and Schwenck [1917]. The filtrate from the trichloroacetic acid precipitation was distilled with 10 % sulphuric acid, while water was dropped in to keep the concentration of sulphuric acid constant. All trioses were thus converted into methylglyoxal, which was precipitated from the distillate by heating with a solution of *p*-nitrophenylhydrazine. When cold the precipitate of methylglyoxal *bis*-nitrophenylhydrazone was filtered off in a Gooch crucible, washed with cold water, dried *in vacuo* and weighed. The presence of acetaldehyde does not affect the estimation because the *p*-nitrophenylhydrazone of acetaldehyde is soluble in water in the concentrations in which it would occur in the distillate from fermentations.

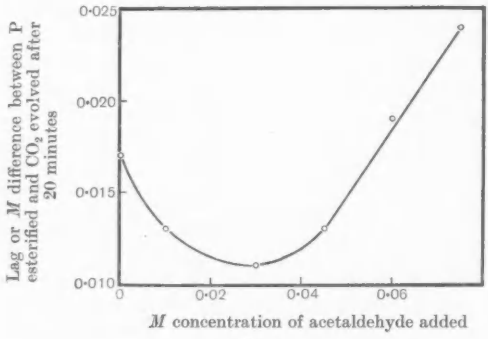

Fig. 1.

That there is an increase in the amount of triose present when the lag in fermentation is at its maximum is shown by the results obtained in Table III. The agreement between the amount of lag and the amount of triose formed is not very exact but the changes are of the same order.

Table III. *The triose present during the period of lag.*

Conditions as in Table I. Results expressed in total millimolecules.

Time after addition of phosphate (mins.)		0	5	10	15	20	30	50
Exp. 180								
Fermenting glucose	Lag	—	0·08	0·11	0·25	—	0·20	0·10
	Triose present	0·16	0·23	0·36	0·34	—	0·25	0·21
	Additional triose formed	—	0·07	0·20	0·18	—	0·09	0·05
Exp. 199								
Fermenting fructose with acetaldehyde	Lag	—	0·14	0·16	0·10	0·04	—	—
	Triose present	0·09	0·19	0·17	0·11	(0·25?)	0·10	—
	Additional triose formed	—	0·10	0·08	0·02	(0·16?)	0·01	—

Although the method of distillation with dilute sulphuric acid yields methylglyoxal in the distillate, methylglyoxal itself does not appear to be present in the trichloroacetic acid filtrates from fermentations. Methylglyoxal is precipitated by dinitrophenylhydrazine in cold acid solution, but the filtrates from fermentations never give the methylglyoxal derivative with this reagent in the cold. If dinitrophenylhydrazine is added immediately after removal of the proteins, a precipitate of acetaldehyde dinitrophenylhydrazone (M.P. 151–152° after recrystallisation from pyridine and ether) is obtained. But, if the trichloroacetic acid filtrate is allowed to stand for more than an hour before the addition of the hydrazine, no precipitate is obtained. Thus even acetaldehyde changes in the solution and it is possible that methylglyoxal, which is still more reactive, changes almost immediately to some compound, possibly a polymer, which is reconverted into methylglyoxal on distillation with acid.

Kluyver and Struyk [1927] suggest that a triosephosphoric ester is formed in fermentation. Such a compound might not be precipitated by hydrazines, yet yield methylglyoxal on distillation with acid. There is, however, no definite support for the existence of such a triosephosphoric ester.

IV. Oxidation-reduction potentials of yeast preparations.

If acetaldehyde acts as an oxidising agent or hydrogen acceptor in fermentation, it must have a definite apparent oxidising potential. It cannot have a true oxidation-reduction potential, because it is reduced irreversibly. The reduction is not a simple electronic change and the reduction product, alcohol, is a feeble reducing agent in aqueous solution. The apparent potential of acetaldehyde was determined by adding it to solutions of leuco-dyes of known oxidation-reduction potential in buffered phosphate solution at p_H 6·0. In this way acetaldehyde was able to oxidise reduced indigodisulphonate, methylene blue and thionine, so that its oxidation potential at p_H 6·0 must be higher than 0·10 volt. Acetaldehyde will not oxidise potassium ferricyanide or the leuco-compounds of 1-naphtholindophenol-2-sulphonic acid and 1-naphtholindo-2:6-dibromophenol-2-sulphonic acid, so that its oxidation potential is probably below 0·15 volt. These determinations were carried out at p_H 6·0 because this was found to be about the p_H of fermenting yeast preparations containing the usual amount of phosphate and saturated with carbon dioxide.

The p_H of fermentation was determined by use of the glass electrode. The reaction of phosphate with yeast juice fermenting fructose was carried out in the cup of a glass electrode similar to that described by Bayliss, Kerridge and Verney [1926]. The readings over 40 minutes during the reaction varied from p_H 6·2 to 6·3. This result is in agreement with the results obtained by Biilman and Katagiri [1927] for phosphate solutions containing glucose and alcohol and saturated with carbon dioxide. These workers used the quinhydrone electrode, which cannot be applied to yeast solutions on account of their oxidation-reduction potential.

Although glucose is more strongly reducing than fructose at p_H 7·5–9·0 [Wurmser, 1927], in solutions as acid as p_H 6·0 it is doubtful if glucose and fructose have any oxidising or reducing power. In buffered phosphate solution at p_H 6·0 neither glucose nor fructose could reduce methylene blue or oxidise leuco-naphtholindophenoldisulphonate in 12 hours at 30°. It is therefore improbable that the different behaviour of glucose and fructose in fermentation with free phosphate is due to difference in oxidising or reducing properties. The difference in behaviour possibly lies in a difference in the ease of esterification of the two sugars.

Cannan, Cohen and Clark [Clark and collaborators, 1928, p. 306] had found that the oxidation-reduction potential of suspensions of washed yeast cells at p_H 5·75 fell from 0·10 to − 0·05 volt in 5 hours. Having determined that the p_H of yeast preparations remained steady during the reaction of phosphate, it became possible to determine the oxidation-reduction potential by adding dyes, and noting the time necessary for decoloration. The acetone precipitate of yeast juice, being free from hexosephosphatase, was very useful for this determination of the oxidation-reduction potential of fermentation with free phosphate, because no fermentation or reduction of dyes occurred until free phosphate was added. The time taken for the reduction of various dyes added in minimal concentrations (0·01 %), so as to have as little poising effect as possible, are shown in Table IV. The figures for the approximate E_0' (the E_h at which the dyes are 50 % reduced) at p_H 6·0 are taken from papers by Clark and his collaborators [1928]. The results show that the oxidation-reduction potential falls from 0·10 to − 0·03 while the greater part of the reaction with phosphate occurs.

Table IV. *The time taken for the reduction of dyes added to the acetone precipitate of yeast juice fermenting glucose in the presence of* K_2HPO_4.

Dye added (0·01 %)	E_0' at p_H 6·0	E_h for 98 % reduction	Time for reduction (mins.)
1-Naphtholindo-2:6-dibromophenol-2-sulphonate	0·19	0·14	2
1-Naphtholindophenol-2-sulphonate	0·18	0·13	5
Thionine	0·10	0·05	10
Methylene blue	0·05	0·00	15
Indigotetrasulphonate	0·00	− 0·05	30
Indigotrisulphonate	− 0·03	− 0·08	Partially reduced
Indigodisulphonate	− 0·07	− 0·12	Not reduced

Experiments were next carried out with higher concentrations of the dyes so that they exerted some poising effect on the system. In this way the rate of fermentation in the presence of free phosphate was measured at different oxidation-reduction potentials. Results of such experiments are shown in Table V. It will be seen that dyes more strongly oxidising than thionine or more strongly reducing than indigodisulphonate do not accelerate the fermentation, but in addition to methylene blue both thionine and indigotetrasulphonate act as hydrogen acceptors. The failure of indophenol dyes to increase fermentation may be due to their quinone structure, so that they

might combine with the protein of the enzyme, or to the phenolic structure of their reduced forms so that they might inhibit fermentation in the same way as phenol. Both quinone and quinol hinder fermentation. The dyes, however, do not seem to combine in any way, as on addition of potassium ferricyanide at the end of fermentation the colour of the dye is always restored. Potassium ferricyanide, a different type of compound and a stronger oxidant than any of the dyes used, is reduced in fermentation but the fermentation is not increased.

Table V. *The effect of different hydrogen acceptors and reducing agents on fermentation.*

1 cc. 2*M* K_2HPO_4 added to 2 g. acetone precipitate of yeast juice in 20 cc. 10 % glucose solution.

Substance added	E_h during reaction (approximate)	Millimolecules CO_2 evolved in 20 minutes
None	0·00?	0·16
0·05 % indigodisulphonate	− 0·03	0·10
0·05 % indigotrisulphonate	− 0·02	0·10
0·05 % indigotetrasulphonate	− 0·01	0·52
0·05 % methylene blue	0·01	0·47
0·05 % thionine	0·03	0·40
0·05 % naphtholindo-2:6-dibromophenol-2-sulphonate	0·14	0·18
0·05 % naphtholindophenol-2-sulphonate	0·13	0·10
0·05 % acetaldehyde	—	0·86
Leuco-dyes		
0·05 % leuco-indigodisulphonate	− 0·10	0·08
0·05 % leuco-methylene blue	0·00	0·42
0·05 % leuco-naphtholindophenol-2-sulphonate	0·12	0·12

The effect of adding leuco-dyes throws some light on the mode of action of dyestuffs in fermentation. Leuco-indigodisulphonate almost stops fermentation, but leuco-methylene blue accelerates fermentation to nearly the same extent as the oxidised dye and leuco-naphtholindophenolsulphonate has no effect on the fermentation. As reduction does not therefore have any great effect on the action of dyes, it is probable that they act as hydrogen carriers rather than as hydrogen acceptors. Thus it might be expected that methylene blue would be reduced by methylglyoxal and oxidised again by acetaldehyde (the last reaction can be shown to take place in the test-tube without enzyme). If this is the case, only those dyes which can be oxidised by acetaldehyde and reduced by methylglyoxal will be active in fermentation and the apparent reducing potential of the active methylglyoxal of fermentation should be about − 0·02 volt at p_H 6·0.

The action of acetaldehyde in accelerating the reaction of phosphate is probably different from that of dyes. The reduction of aldehyde differs from that of the dyes which accelerate fermentation in not being reversible, so that acetaldehyde acts purely as hydrogen donator, not as hydrogen carrier. It is probably more effective in fermentation because of some specificity of the oxidase for acetaldehyde.

SUMMARY.

1. The lag between phosphate esterification and carbon dioxide evolution, in fermentation by yeast preparations, is not removed by the addition of those hydrogen acceptors which reduce the time necessary for the attainment of the maximum phosphate rate of carbon dioxide production.

2. The lag is accompanied by an increase in the amount of triose present; it is possible that accumulation of methylglyoxal prevents further esterification of phosphate.

3. During the reaction of phosphate in fermentation the p_H of the solution, saturated with carbon dioxide, remains constant between 6·2 and 6·3.

4. Those dyes which accelerate fermentation by acting as hydrogen acceptors or carriers have an E_0' between 0·00 and 0·10 volt at p_H 6·0; it is probable that they must be reducible by methylglyoxal and oxidised again by acetaldehyde.

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LXXXI. CARBOHYDRATE METABOLISM IN BIRDS.

II. BRAIN LOCALISATION OF LACTIC ACIDOSIS IN AVITAMINOSIS B, AND ITS RELATION TO THE ORIGIN OF SYMPTOMS.

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In a recent paper [Kinnersley and Peters, 1929] it was shown that opisthotonus symptoms due to avitaminosis B_1 in pigeons were associated with an increase of lactic acid in the brain. The provisional conclusion was reached that the increased lactic acid was specifically due to lack of vitamin B_1 in the brain. If this conclusion be true, not only does it provide cogent evidence for the relation between vitamin B_1 and some phase of carbohydrate metabolism, but we feel that it is evidence of chemical change underlying the activity of the central nervous system. We have therefore analysed the problem further. It was conceivable that the changes in the brain were merely due to a general increase of lactic acid throughout the tissues. If this compound was present especially in one region of the brain, it would show that there was no direct relation between the increased blood-lactic acid and that in the brain. Further, it would be suggestive evidence for a correlation of the symptoms with a localised chemical abnormality and would indicate the portion of the brain responsible for them. Some preliminary experiments upon this question have already been published [Kinnersley and Peters, 1930].

Experimental.

The treatment of the brain tissue and the methods of analysis have been described elsewhere. No significant changes have been made. Use has been made of the "guillotine" method. The two halves of the brain have been immersed in liquid air within 5 seconds approximately, the time of immersion being stated in the tables. Freezing, though instantaneous at the cut surface, must take some seconds for completion throughout the mass. When removed from the liquid air, the brains are too hard to divide. Fortunately, we have found that thawing produces little change, if any, in brain tissue which has been frozen in liquid air. We have previously quoted two experiments in support of this [Peters, 1930]. Further experiments which illustrate the same point are shown in Table I, Exps. 1, 2 and 7. Allowing the brain tissue to thaw completely has not led to large increases in lactic acid content. In one case (Exp. 7) there would seem even to have been a decrease of the lactic acid. We do not, however, lay stress upon these changes. They indicate that brain tissue can

probably be allowed to thaw completely without much change in content of lactic acid. In practice, the required parts of the tissue have been removed before being completely thawed, to minimise any possibility of change in the abnormal tissues. About 15–20 minutes after removal from the liquid air the tissue can be cut with a knife. At this stage portions were removed with a clean, cooled scalpel and dropped into ice-cold 10 % trichloroacetic acid; any congealed blood was cleaned from the split half of the brain before selecting the parts. As the significance of the experiments depends upon comparative rather than upon absolute values, great care has been taken to work under strictly comparable conditions throughout.

Parts of the brain selected. These have been in various experiments: (1) the cerebrum, (2) the cerebellum, (3) the mid-brain with the optic lobes, and (4) the remainder, bulb and medulla. Various combinations of these have been used. By cerebrum we mean here the parts of the brain included by a horizontal cut parallel with the lower surface of the cerebral hemispheres, and in the sections of mid-brain have been included the optic lobes together with such tissues as lie in the median plane upon a horizontal line drawn approximately parallel with the lower surface of the optic lobes. The approximate weights are for the cerebrum 0·96 g., cerebellum 0·27 g., mid-brain and the remainder 0·43 g.

"*Sugar values.*" These have been obtained upon blood by the Hagedorn and Jensen method, using zinc precipitation as described by these authors. Values for "sugar" by the Hagedorn and Jensen method, obtained upon trichloroacetic acid filtrates, are usually higher than these, but are not consistently so. As shown recently by Gulland and Peters [1930], these differences are due to varying amounts of glutathione and are not so reliable as the direct zinc precipitation. In any case, values would have to be reduced by some 70 mg. per 100 cc. to obtain the true "sugar" value.

Determination of lactic acid. The following is a description of the method actually used. The samples of brain tissue were placed in tared 25 cc. centrifuge tubes containing about 5 cc. of 10 % ice-cold trichloroacetic acid to 1 g. of tissue; the tubes were quickly re-weighed to obtain the weight of the tissue. It was minced with a glass rod and left to extract in the trichloroacetic acid with the addition of an equal volume of water for 30 minutes or longer and was then centrifuged and filtered. Fresh trichloroacetic acid (2–3 cc.) was added to the residue, which was ground and allowed to stand for re-extraction for at least 30 minutes. The latter operation was repeated. Water was added and the tube centrifuged. The combined centrifugates made a total volume of about 20 cc. It was not necessary to leave the tissue to stand for 24 hours as has been claimed recently by Jungmann and Kimmelstiel [1929]. The combined centrifugates were divided into two (or in later experiments into three) parts and each of these was separately treated with 1 cc. of 10 % copper sulphate and sufficient $Ca(OH)_2$ suspension to make it alkaline (about 4 cc.). By performing these extractions separately, it has been our belief that a more proper control is made of the errors produced in the copper-lime treatment. After

standing, the mixtures were centrifuged, and the clear supernatant fluid was used for determination of lactic acid by the method of Friedemann, Cotonio and Shaffer [1927].

Accuracy of the lactic acid determinations. In the case of blood, where determinations have been made upon trichloroacetic acid filtrates, duplicate values have always agreed remarkably closely, to within ± 0·01 cc. of *N*/100 iodine. This means that the values given in the tables are the average of two samples not differing from one another by more than ± 0·5 mg./100 g. tissue. For the brain tissue, all the values given in the tables are the mean of two duplicates which did not differ by more than ± 0·02 cc. (approximately ± 0·01 mg. lactic acid) and were often identical. Occasionally we have found quite wide and inexplicable variations between duplicates carried out by our method of separate precipitation at the copper-lime stage, which we are satisfied are not introduced by the reagents. In order to make clear the maximum error allowed in the calculations, it may be said that with the larger amounts of tissue, *i.e.* estimations of cerebrum and cerebellum taken together, or upon the remainder, the values given in the tables will lie within ± 2 mg./100 g. tissue of the figures actually obtained. More generally the variation is much less. But where smaller amounts of tissue only were available the error is proportionately greater.

The method of Friedemann, Cotonio and Shaffer in our hands gave a blank value upon the reagents of 0·05 to 0·06 cc. *N*/100 iodine, and a yield of 95 to 97 % when tested against recrystallised zinc lactate. Lactic acid values in the tables have not been corrected for this difference [see Friedemann, 1928].

The substance estimated. The amounts of lactic acid concerned are too small to estimate directly as the zinc salt; there is in any event doubt as to whether isolation of a zinc salt can be relied upon more than the latest oxidation methods [see Friedemann, 1928]. We have made the assumption that the substance producing aldehyde is lactic acid. Justification for this assumption can hardly be questioned in the case of the normal brains. Lactic acid itself was isolated from human brain many years ago by Thudichum [1884] and McGinty and Gesell [1925] have isolated lactic acid from the brains of dogs. It was possible, however, that the abnormal brains contained some substance other than lactic acid yielding aldehyde. It would be evidence against this if it could be shown that the aldehyde-producing substance was ether-soluble. The following experiment was therefore carried out. The brains of avitaminous pigeons were taken soon after death, extracted as usual with trichloroacetic acid, and an aliquot sample analysed after treatment with copper-lime. The remainder was then saturated with NaCl, 0·5 cc. of conc. H_2SO_4 added, and the whole extracted with ether (specially purified) in a Clausen extractor. The ether filtrates were treated with 10 cc. water, powdered talcum was added and NaOH to alkalinity, and the ether removed upon the water-bath. Portions were taken for direct estimation of lactic acid and also after copper-lime treatment. Under these conditions extraction with ether is slow.

Details of experiment. Two avitaminous brains taken; weight 4·12 g. Trichloroacetic acid extracts analysed. (A.)

	(mg.)	(mg.)
Lactic acid present initially in A...		2·25
„ in first extraction with ether 3 hours	1·88	
„ in second extraction with ether 3 hours	0·22	
„ still present in aqueous phase after the extractions	0·14	
Total ...		2·24

In the two ether extractions therefore at least 95 % of the substance estimated was found to be ether-soluble. The excellent balance obtained shows that nothing was added in the process of extraction. This experiment makes it very probable indeed that the substance concerned in the abnormal brain is actually lactic acid [Friedemann and Kendall, 1929].

Normal brains. Table I gives the results for normal birds. Exps. 1 and 2 are the averaged results for two separate groups of five birds. The remainder represent determinations upon single birds. The lactic acid found within the time stated is approximately the same for the cerebrum, cerebellum and the rest of the brain, and amounts to some 50–60 mg. per 100 g. of tissue except in the case of the damaged brain (Exp. 7 (*a*)).

Reducing values were also obtained and showed in each case a diminution for the (*b*) sample as compared with the (*a*), but this varied from 9 to 40 mg./100 g. Throughout the work reducing values have been obtained, but they are omitted here because they show no consistency and add nothing to those previously published. The figures for brain tissue represent mg. of lactic acid per 100 g.; for blood, mg. of lactic acid and sugar per 100 cc.

Table I. *Normal birds.*

Time means interval between death of the brain and immersion of the material in liquid air

Exp.	Time (secs.)	Cerebrum	Cerebellum	Rest	Notes
1	(*a*) 11	56	46	55	5 birds used. One-half (*a*) of each brain dropped into liquid air at 11 secs., and the other (*b*) at 83 secs. (*a*) and (*b*) averaged results for each set of half brains
	(*b*) 83	93	85	92	
2	(*a*) 11	52	53	58	5 birds used as in Exp. 1, but the brains allowed to thaw in air for 30 mins. longer than Exp. 1
	(*b*) 86	87	94	85	

Exp.	Time (secs.)	Cerebrum and cerebellum	Rest	Blood-lactic acid	Blood-sugar	Notes
3	5	39	43	14	251	
4	4½	56	56	17	210	
5	4	57	59·5	24·5	233	
6	4½	54	51	25	184	
7	(*a*) 5½	70*	64	33·5	221	*Brain badly damaged by knife
	(*b*)	57	53	—	—	
8	3½	54	58	13	242	"Urethanised"

Our standard technique therefore gives for normal brains similar values for lactic acid in different parts of the same brain.

Opisthotonus brains. Table II shows that in pigeons suffering from opisthotonus due to vitamin B_1 deficiency, there is an increase of lactic acid in the

lower parts of the brain as compared with the upper. The deficiency was induced by rice feeding by the usual methods employed in this laboratory.

Table II. *Opisthotonus brains. Acid mg./100 g.*

Exp.	Time (secs.)	Cerebrum	Cerebellum	Rest	Blood-lactic acid	Blood-sugar	Hours symptoms
1	6½	90	94*	124	170	164	?
2	6	65	64*	108	54	357	4
3	6	113	80*	157	—	336	6
4	4	80	85	104	31	246	1½
5	6	87·5	92	137	38	231	1½
		Cerebrum and cerebellum					
6	4–5	63		—	65	283	?
7	5	52*		86	65	405	3
8	5	75		104*	161	177	5
9	5	99		86	53	212	24†
10	7	100		123	62	262	?
11	4	103		113	66	226	2
12	4	89		98	41	270	—
			Mid-brain	Rest			
13	4	61*	52	71*	23	284	2+
14	6	98	114*	124	86	343	—
15	5	76*	105	—	52	—	4
16	4	107	127	113	84	307	—
17	3½	74	87*	98	52	231	2+

* One estimation only.

† Part of mid-brain included accidentally in cerebrum.

Last column indicates the number of hours during which symptoms had persisted.

With one exception, viz. Exp. 13, the blood-lactic acid is high and there is a general tendency for the brain-lactic acid to follow it, though this is not the absolute rule (Exps. 1 and 8). There is only one case, Exp. 9, in which the cerebrum and cerebellum contain more lactic acid than other parts of the brain; in this it was known that some of the mid-brain was accidentally included in the cerebral values. Exps. 13–17 show that as a general rule there is more lactic acid in the lowest section of the brain analysed. The localisation of this acid and its uneven distribution are suggestive evidence that the acid found does not merely accumulate from the blood stream.

In attempting to correlate the "lactic" acidosis with the origin of the symptoms, we examined the condition of the brains in birds with threatening or incompletely developed symptoms. In our previous paper [Kinnersley and Peters, 1929] we showed that rice-fed birds had a normal brain-lactic acid even after some 20 days upon the diet. In one case, however, which could be presumed to be approaching symptoms as it had been upon the diet longer than the others (27 days) we found actually a slight increase. In Table III we have collected a series of results upon birds which in our opinion were threatened with symptoms. The results are somewhat variable as would be expected. One reason for this is that in spite of an extensive experience, it is not easy to be certain in every case how near a bird is to "opisthotonus."

Table III. *Threatening opisthotonus.*

Exp.	Days on diet	Time (secs.)	Cerebrum and cerebellum	Mid-brain	Rest	Blood-lactic acid	Blood-sugar
1	20	3–4	69		79	45	279
2	24	4	—		75	44	234
3	17	5	64		72	67	246
4	22	—	41		72	67	292
5	27	7	62		97	78	269
6	30	4–5	57		73	—	—
7	32	6–7	55		63	21	242
8	12	4	66		96	87	260
9	—	5	48	52*	60*	14	244

* One estimation only.

The results are within normal limits for cerebrum and cerebellum, but raised for the lower part of the brain. If we exclude the two experiments showing a value for the lactic acid in the "rest" as high as 97 mg., the remaining values all lie under 80 mg. per 100 g. Practically all therefore are below those of Table II. Exps. 7 and 8 are within the normal limits for lactic acid in blood and brain. It is very probable that we had here selected birds which were not sufficiently advanced to show the brain condition. This is supported by the low blood-lactic acid. The facts are therefore that in the avitaminous bird, lactic acid begins to increase in the lower parts of the brain before the symptoms of opisthotonus appear. When it reaches a certain value (under the conditions of our experiments some 80 mg. per 100 g.) opisthotonus tends to arise.

The presence of opisthotonus is associated with an increase in the amount of lactic acid present; such increases are again unevenly distributed. A rise in lactic acid usually takes place in all parts of the brain, but this is most marked in the lower regions. The rise in the cerebellum is not more than that in the cerebrum. In the experiments in which the mid-brain and "rest" have been analysed separately, there tends to be more in the "rest," but this is not without exception, though not too much weight can be attached to such differences as appear owing to the experimental error involved in handling these small amounts of tissue. Together with the uneven increases in the brain, there occurs an increase in lactic acid in the blood. The increases in brain-lactic acid, however, do not follow the increases in blood-lactic acid directly. This suggests that the two are really independent, which would also follow from the uneven distribution of the lactic acid in the brain. Before, however, being able to decide that this is so, another possibility must be considered. It has been suggested to us that the uneven distribution of lactic acid might be merely the result of some structural peculiarities in the brain tissue, which lead to irregular adsorption from an increased blood-lactic acid, or to irregular elimination of lactic acid from the tissue. As we have had no success with attempts to raise the blood-lactic acid by intravenous injections of lactic

solutions in normal, unanaesthetised birds, we have had recourse to the expedient of flying birds round a room for periods of 30 minutes, and then taking the brains for analysis (Table IV).

Table IV. *Exercised birds.*

Exp.	Time (secs.)	Cerebrum and cerebellum	Rest	Blood-lactic acid	Blood-sugar
1	5	62	60	80	237
2	4–5	71·4	73	50	237
3	4	93*	89*	121	184
4	3½	73	79·5	126	219
5	4	81	83	86·5	295

* Poor duplicates.

Table IV brings out several points. Exercise increases the blood-lactic acid as would be expected, bringing it in fact to a level even above the amount usually found in avitaminosis. Together with this increase, when the bird is exhausted (Exps. 3, 4 and 5) there appears in the brain an amount of lactic acid as high as that in the bird with symptoms of threatening opisthotonus; but it is evenly distributed. The lactic acid in the brain does not increase above 90 mg. even with enormously high blood-lactic acid. The localised nature of the chemical lesion in vitamin B_1 deficiency responsible for the appearance of increased lactic acid in certain parts of the brain lends support to the conclusions of Fisher and Peters [1930] that the condition is not caused by impairment of brain circulation. It is associated with the initiation of symptoms of a peculiar type which are cured by administration of vitamin B_1, concurrently with the return of brain-lactic acid to a more normal value. In our opinion the results indicate that there is some missing factor in the tissue. Since this change is ameliorated by giving vitamin B_1 (most speedily of all by the intracranial injection of torulin [Peters, 1930]), lack of vitamin B_1 in the tissue leads to the accumulation of lactic acid in that tissue. This appears to be therefore a proof of the connection of vitamin B_1 with the intermediary metabolism of carbohydrates, a view which has been often mooted [Braddon and Cooper, 1914; Funk and v. Schönborn, 1914; Randoin and Lecoq, 1927].

We are upon less certain ground when we attempt to guess the exact function of torulin from the presence of the excess lactic acid. There is no evidence that the glycolytic power of the tissue is impaired, or that the powers of synthesis of the lactic acid precursor are diminished. In experiments upon birds showing symptoms, we have found that the brain-lactic acid maximum, after allowing the tissue to stand for 90 seconds before freezing, was in one case for cerebrum and cerebellum 142 mg. and for the remainder 150 mg., the blood-lactic acid being 56 mg., and in another case for cerebrum, etc. 178 mg., and for the rest 230 mg. The lactic acid maxima are much greater than normal and are not necessarily different for different parts of the brain in the deficient birds. There must be therefore more precursor in these brains than in the normal. This is curious and as yet unexplained. Insulin lowers the lactic acid

maximum for brain and vitamin B_1 deficiency raises it. These facts must be reconciled with any general theory of the condition. The evidence against the view that the increased lactic acid is due to an enhanced rate of glycolysis which has been presented elsewhere is still valid. Upon these grounds we think that vitamin B_1 is concerned with the oxidative removal of lactic acid, though admitting that a substantive proof of this is at present lacking.

We are aware for instance that the appearance of the acid might be secondary to some change in p_H of the tissue induced by lack of vitamin, or that the function of vitamin B_1 might even be to stimulate the production of some missing hormone, which then activates the tissue. In the latter case it seems to be more difficult to explain the localisation.

It must be emphasised that the figures in the above tables, though strictly comparable, do not represent the initial state of the "resting" brain-lactic acid.

Further considerations. In the above experiments, the use of anaesthetics or similar substances has been avoided in order to minimise any complication produced by their results. It must not be forgotten that substances like urethane are well known to disturb surface reactions, and that they inhibit the action of the cell dehydrogenases [Keilin, 1929]. However, since Cobet [1929], in work which only came to our notice when the major part of this research was completed, has found that brain-lactic acid in deep urethane anaesthesia is very low, we have carried out a few experiments upon this point. Cobet found that in the case of rabbits the resting values for lactic acid (by a colorimetric method) were 10–12 mg./100 g., increasing very much upon injury. The pigeons employed by us were deeply urethanised. After having been left undisturbed for periods of 30 minutes, the birds were guillotined and the samples of tissue frozen as usual. Table V gives the results.

Table V. *Lactic acid in brains of "urethanised" animals.*

Exp.	Condition	Time (secs.)	Cerebrum and cerebellum		Rest	Blood-lactic acid	Blood-sugar
1	Normal	3½	54		58	13	242
2	,,	3½	46		60	18	237
3	,,	3½	80		73	11	228
4	,,	3½	52		68	13	274
5	Opisthotonus	5–6	82		101	40	249
			Cerebrum	Cerebellum			
6	,,	4	64	80	87	14	253
7	,,	4	72	62	91	26	261

Our "urethane" results in no case approach those of Cobet. It seems to be impossible even with rapid work in pigeons to reach his low limits, though the degree of injury was certainly no more in our experiments. Inspection of Table V shows that the normal urethanised pigeons show most inconsistent values for brain-lactic acid, but have a low blood-lactic acid. Results for avitaminous birds are the same as usual.

Iodoacetic acid. In a recent publication Lundsgaard [1930] has shown that animals poisoned with monoiodoacetic acid die in *rigor* without showing any rise of lactic acid in the muscles. The muscle is fixed as it were in the resting state [see also Meyerhof, 1930]. Table VI shows the amounts of lactic acid found in pigeons killed by intravenous injections of the poison. Injections were made into the wing vein and varied from 50 to 100 mg. of the compound. Brains were removed in the usual manner and immersed in liquid air as soon as possible after the death of the animal, which took place at times varying from 2 to 15 minutes after injection. The time elapsing between injection and death apparently did not influence results.

Table VI. *Pigeons killed by iodoacetic acid.*

Exp.	Condition	Time of death (mins.)	Cerebrum and cerebellum	Rest	Blood-lactic acid	Blood-glucose
1	Normal	4	24·5	24·7	7·9	217
2	,,	8	29·5	29·5	6·6	214
	,,	2	27·5	35	—	—
3	H.R.	5	34	47	26·6	214
4	,,	15	36	56·5	81	231
5	,,	7½	53	75	81	272

These results are of great interest, being the lowest which we have obtained for normal and avitaminous pigeons' brains. It will be noticed that the "opisthotonus" birds all show higher lactic acid, and that in these the lower part of the brain is again 13–22 mg. higher than the values for the cerebrum and cerebellum. These amounts bear roughly the same relation to one another as do those of Tables I and II. Until more evidence is forthcoming as to the exact action of monoiodoacetic acid, we cannot draw conclusions as to whether the experiments of Table VI represent the lactic acid content of the tissues during life. But the results constitute the strongest additional support for the views put forward in the earlier part of this paper, and exclude the possibility that the high lactic acid of the avitaminous brain is due to increased rate of glycolysis.

Relation of the symptoms to the presence of lactic acid.

If the experiments in Tables II and III are examined, it will be noticed that in practically every case in which the cerebrum and cerebellum were separately analysed, the values were identical within the limits of experimental error. They were also always lower than the value for the rest of the brain. In Exp. 2, Table II, the values for cerebrum and cerebellum are actually within normal limits. In the experiments of Table III the fact that often immediately before symptoms appear the values for cerebrum and cerebellum analysed together are within normal limits indicates that the cerebellum is sometimes normal (so far as lactic acid is concerned). This suggests that the symptoms are associated directly with the presence of lactic acid in the lower parts of the brain. Certain other facts support this. Fisher and one of us in

another connection have repeatedly removed the cerebrum and basal ganglia under ether anaethesia in avitaminous birds. As soon as the bird recovers from the anaesthetic, the symptoms reappear. They cannot be due to release of cerebral control, because it is well known that decerebration alone makes little difference to the appearance of a pigeon. This confines the problem to the lower part of the brain, and to the cerebellum. Now the removal of the latter induces a similar condition to the "opisthotonus" of avitaminosis. The matter has been lately studied carefully by Bremer and Ley [1927].

Symptoms are induced by removal of the cerebellum, and hence the problem of their origin in avitaminosis reduces either to (1) increased stimulation of the mid-brain, etc., or (2) to release of cerebellar control. Since we have found cases in which symptoms are present with a practically normal "cerebellar"-lactic acid, any possible release of cerebellar control cannot take place by a lactic acid block in the cerebellum. So the symptoms must be associated with increased lactic acid in the lower parts of the brain. The condition produced would appear to be the "brain" analogue of *rigor* in the muscle. Activity of the brain tissue concerned produces lactic acid which then tends to accumulate because it is incompletely removed. When a certain concentration is reached, opisthotonus appears. This would be consistent with the common experience that symptoms can be readily induced by exercise when avitaminosis is sufficiently advanced. It is of course impossible upon the evidence here presented to decide whether the lactic acid acts by inhibition or by stimulation.

Visual inspection of the parts especially affected shows that they appear white, whereas the cerebrum appears grey. This difference in appearance may possibly underly the special susceptibility of the lower parts of the pigeon's brain to lactic acidosis. We do not feel that there is at present any justification for thinking that vitamin B_1 deficiency affects conducting rather than cell tissue, because Dr Carleton[1] finds that the whitish-looking mid-brain tissue contains a large proportion of cells. The matter evidently warrants further investigation.

Discussion.

We recognise that the view which has been advanced as to the origin of these symptoms in avitaminosis does not appear to be consistent with our own experiments in Table IV upon birds exhausted by exercise. These certainly show that lactic acid can accumulate without inducing the syndrome in question, but it is felt that a general condition of this nature cannot fairly be compared with a localised affection.

It must be remembered that ether anaesthesia in the avitaminous bird will abolish the symptoms, without altering the changes in the lactic acid. While the condition here investigated is a concomitant of lactic acid accumulation, this is certainly not the case with other similar symptoms. Insulin

[1] Personal communication.

convulsions in the pigeon (as has been shown by Holmes and Holmes for the rabbit) are associated with a low brain-lactic acid not varying in amount in different parts of the brain. It is impossible to bring into one scheme all the various conditions (apart from surgical lesions) which will induce this condition in the pigeon, without having recourse to some conception such as that of the "Meyerhof cycle" [1926] for carbohydrate metabolism. With the idea, however, that maintenance of the activity of the tissue depends upon a cyclical chemical process, it becomes possible to understand how changes of varying type may result in the same interference with function. This matter has been discussed elsewhere [Peters, 1930].

In our previous paper, we drew attention to the discrepancy between our results and the earlier view of Holmes and Holmes [1925, 1926 and 1927] that lactic acid in the brain arises immediately from the blood-sugar. Since then these authors have kindly drawn our attention to a later publication [1928] in which this view is to some extent modified. We feel that the above results can only be interpreted upon the view that there is a precursor of lactic acid in the tissue itself, however much this may in turn be in equilibrium with the blood-sugar.

Summary.

1. The increased lactic acid found in the brains of pigeons showing symptoms of opisthotonus due to avitaminosis B_1 is localised especially in the lower parts of the brain.

2. In the period of rice feeding during which symptoms are threatening, lactic acid is increased only in the lower parts of the brain.

3. The localised character of the chemical lesion proves that vitamin B_1 is associated with the intermediary metabolism of carbohydrate.

4. Symptoms are due to the accumulation of lactic acid in the lower parts of the brain.

5. The lowest lactic acid found in the brain of a normal pigeon was 24 mg. per 100 g. of tissue, after death by injection of monoiodoacetic acid.

6. Severe exercise may cause a rise in the lactic acid content of the brain, which is distributed evenly over the tissue.

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LXXXII. THE METABOLISM OF DEVELOPING SALMON EGGS.

I. THE SIGNIFICANCE OF HATCHING AND THE RÔLE OF WATER IN DEVELOPMENT.

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Material.

Eggs of the Atlantic salmon (*Salmo salar*) were procured in the eyed stage and maintained in a small hatchery at a temperature of 8 to 10°. Younger stages were not reared in the laboratory, for although many could be kept alive for some weeks after the journey from the hatchery there was no further development. The series of analyses given is that of a group of 5000 eggs which came to the laboratory in the eyed stage and were from one female. Fifty specimens were taken for each test and the adherent moisture removed between sheets of filter-paper. They were then weighed in a dried extraction thimble supported on cotton wool in a tared weighing bottle. This was the wet weight. The eggs and larvae were dried to a constant weight (the dry weight) at 100°.

Obvious changes during development.

Experts in fish culture recognise several critical periods in the development of salmonid eggs. For a day or two after fertilisation they are hardy and can be moved about and arranged in the hatchery trays without injury; after this they become exceedingly delicate until the appearance of pigmented eyes within the shell. The first period is one of comparatively undifferentiated cell division, the second that of the organogenesis or morphological differentiation. In the eyed stage very few eggs die and much rough handling is not detrimental. About three weeks before hatching there ensues another period of great mortality, in the phase of histogenesis. Many of the embryos become opaque within the shell while the yolk remains transparent. A large number of such larvae have been hatched and examined. Necrosis usually begins as one or more ring-shaped areas in the tail region from which it spreads rapidly until the whole tail has become opaque. At this stage there is no tail circulation, poor vitelline circulation, feebly and irregularly beating heart and very little movement. No larva hatched in such a condition has recovered. A group of larvae in various stages of opacity was hatched and the "case histories" followed in

detail. Extracts from two typical examples are given. The embryos began normally to hatch about Jan. 15.

Case 1. Hatched Dec. 29. Physical conditions; dorsal fin sloughed into a large mass of necrosed tissue; adipose fin had dropped off leaving only a jagged edge; two ring-shaped opaque spots, one situated just posterior to the pectoral fin and the other under the adipose fin; heart rate 84 per minute (control heart rate 94); good circulation to all parts of the body. On Dec. 30 the dorsal fin sloughed off and from then on the fish began to recover, until on Jan. 9 there was no trace of the white patches, the heart rate was normal, and the fish very active.

Case 2. Hatched Dec. 30. Physical conditions; whole of tail half way to adipose fin enclosed in a white sheath of necrosed tissue with a large flap hanging loose; a second small patch under the adipose fin; heart rate 69 per minute (control heart rate 84); poor embryonic circulation and no blood supply over distal parts of yolk sac; very little activity. Hatching produced a slight temporary improvement, but after Jan. 2 the heart rate decreased and the general condition became worse until death on Jan. 6. The sudden temporary improvement after artificial hatching has been noted in most cases.

On Jan. 2, 130 typically affected eggs were selected and divided into two groups. Those of one group were hatched artificially by tearing the shell with fine forceps. Both groups were kept under similar conditions to determine the effect of hatching on the fatality of the condition. The final results were the same, 11 out of the 65 surviving in each case. Natural hatching had taken place before the conclusion of the experiment on Jan. 19. It was noticed that death occurred more quickly in the hatched group, due to the immediate attack of the necrosed tissue by the fungus *Saprolegnia ferax*, while the unhatched larvae might live for some days longer protected by the shell. It is believed that under different experimental conditions, using fungus-free water, the hatched group would have shown a larger proportion of survivors.

If bacteria or mycelia could go through the shell, infect the embryo within, and by destruction of the shell produce hatching, an interesting case of symbiosis would be suggested. To test this idea affected larvae and controls were hatched under aseptic conditions and reared in vials at a suitable temperature, protected from atmospheric contamination. Before hatching any possible infection on the outside of the shell was removed by immersion for one minute in 3 % formalin. Experiments determined that at this age an egg is capable of withstanding an immersion of 3 minutes in 3 % formalin without serious injury. It is believed that an exposure to formalin which would remove infection within the egg would also kill the larva. Controls and some of the experimental animals lived until the conclusion of the experiment some 10 weeks after hatching. Those which died were left in the vials and observed, but there was no decomposition except the usual autolytic one, and examinations of living and dead eggs showed no bacteria or fungus. The conclusion is that it is not from external infection that the larvae die within the shell.

It was found that immersion in formalin of an egg 3 weeks before its hatching time, caused it to hatch within a few hours. Scheminzky and Gauster [1924] showed that trout eggs which are continually exposed during development to the action of a weak electric current hatch a week earlier than the controls. The resulting larvae do not differ in any way from the normal. These authors attribute the result to the destruction of the egg capsule, but it may be due to nothing more obscure than a marked stimulation of embryonic activity.

Wintrebert [1912] has demonstrated the secretion of a protease by the trout embryonic ectoderm which dissolves the shell and permits hatching. It is one purpose of this paper to point out that the phenomena associated with the production of this enzyme have a far greater effect on embryonic metabolism than a plain statement of their final result would indicate. It is necessary for the larva to suspend body-building operations and attend to the problem of blazing a trail to the outside world. The effort profoundly modifies embryonic metabolism and it is not until some weeks after hatching that the uneventful course of development is resumed. The dissolving of a capsule so tough that it is broken down only with difficulty in caustic potash solution [Miescher, 1897] is accomplished by a concentrated protease solution in the perivitelline fluid. Variations in the water content and metabolism, and the frequent death of the embryos within the shell are explained by this developmental modification. The region of the embryo which first begins to die lies in the extremities (tip of tail, fins, etc.), and this further supports the idea that the enzyme directly attacks the embryonic tissue, for it is here that the embryo is most susceptible to the action of unfavourable environmental factors. (Hyman in 1921 exposed *Fundulus* eggs to lethal concentrations of cyanide solutions and found that the tail died first.)

The egg capsule.

The chemistry of the salmonid egg shell is not known in detail. Steudel and Osato [1923] and Osato [1923] concluded from the nature of the amino-acids in herring egg shells that the fish egg is homologous only with the yolk of the bird's egg, while the shell of the latter is a new modification for terrestrial life. The albumin of the bird egg corresponds to the mucilaginous covering outside the shell of amphibian and some fish eggs, and the vitelline membrane is the homologue of the fish egg capsule. (The loose use of the word "chorion" for a fish egg shell suggests a homology which does not exist.) It follows that an aquatic egg shell may take a more active part in the scheme of development than a terrestrial one.

After fertilisation the capsule is pale in colour, of a dull sheen, and relatively thick and brittle. It can be stained intensely black with osmic acid [Scheminzky and Gauster, 1924]. Its strength increases for a time, but before hatching it becomes noticeably thinner, somewhat loose and folded, easily torn, and loses its sheen. Its reaction to osmic acid at this time indicates a

decrease in fat, or at least of the lipins containing unsaturated acids. Shortly before hatching the water increase in the egg causes the shell to become taut. Many eggs may be hatched at this stage by gently squeezing.

Nordgaard [1896] observed that if a dead opaque salmon egg were placed in a salt solution it became transparent. Gray [1920] found that any of the usual ways of killing a trout egg, such as heat, cold, electric shock, physical shock, etc., were followed by exosmosis of the electrolytes and this caused precipitation of the globulins with resultant opacity. The amount of exosmosis under various conditions could be quantitatively measured from the variations in the electrical resistance of the water in which the eggs were dying. The re-solution of the globulins in normal saline demonstrated that under the unfavourable conditions the capsule had become permeable. The temporal relation between exosmosis and globulin precipitation was determined by Scheminzky and Gauster [1924], who found that the electrolytes leave the egg before the globulins are precipitated. Thus the primary injury is to the membrane rather than to the contents of the egg. Now Stockard [1921] and a number of other workers have demonstrated critical stages in embryonic development, at which it is particularly easy to subject an egg to a shock which will later produce an embryonic deformity. These conditions are generally attributed to changing properties of the protoplasm (varying oxidation rates of different parts at different times, etc.), but the final explanation may well be found in the egg membrane. It is worth noting that salmonids are the fish in which embryonic deformities have been most frequently observed in nature.

Scheminzky and Gauster themselves do not believe that the varying susceptibility during development is due to the membrane, because the electric current required to kill an egg in the later stages increases, while the tensile strength of the shell decreases. (Hein [1907] determined the pressure necessary to crush an egg at various stages.) There is, however, no good reason to believe that permeability is determined by strength and thickness. Furthermore, during development a new membrane system comes to surround the yolk. In later stages two membrane systems, a band of muscle and a fluid-filled coelom, must be penetrated in addition to the shell before the yolk is reached. An inspection of Scheminzky and Gauster's fig. 19 suggests that the coelom surrounds the yolk at the 24th day, since the resistance of the egg rises suddenly at this time.

It has been agreed by most workers that the capsule of the living egg is normally permeated by water but not by electrolytic or colloidal substances. Svetlov [1929] has recently sharply criticised this conception. Using micromethods he found a lower osmotic pressure in the perivitelline fluid of trout eggs than in the yolk, and concluded that the egg contents have the power of maintaining their osmotic pressure independently of the capsule. This does not deny the same function to the cell membrane. Kite [1913] found that most animal and plant cells regulate their permeability as much by the cytoplasm as by the cell wall.

Svetlov also found that salt ions passed through the capsule of a living egg and into the perivitelline fluid. Now Loeb [1912] has shown that the action of a solution of one salt on *Fundulus* eggs is to increase the permeability of the capsule. If a solution of two antagonistic salts such as NaCl and $CaCl_2$ were used there was no changed permeability. What Svetlov has really done therefore is to injure the membrane with an electrolytic solution, the ions of which afterwards passed through. Loeb points out that a dilute acid or alcohol solution has the same effect as a salt solution, and he says, "The increase in the permeability of the membrane for water and salts under the influence of acids is due to an alteration of the protein constituents of the membrane." Thorner [1929] immersed developing *Fundulus* eggs in KCl until the heart ceased to beat, and then removed them to fresh water or sea-water. In distilled water there was a slow extrusion of salt through the shell until after some days the heart recovered. The reaction in sea-water was quite different, the time required for recovery being short and directly proportional to the period of exposure to KCl. Since *Fundulus* eggs can develop in either fresh water or sea-water the customary reaction of the shell must be the same in both media. It follows therefore that after immersion in one salt the capsule behaves abnormally. Sumwalt [1929] found that the electrical potential of *Fundulus* eggs was changed to a marked degree if the eggs were immersed in solutions of various salts. In neutral solutions the capsule was more permeable for cations than for anions. Bivalent cations had about half the effect of univalent cations. As the acidity of the solutions was increased the cation and anion permeabilities approached one another until at p_H 3·7 they were equal. In more acid solutions reversal of permeability occurred. Sumwalt reviews the literature on this point which serves to show the pronounced effect of salt and acid on the capsule. Svetlov mentions a simple and convincing proof of the permeability of the capsule to salt under normal conditions. If a very young egg, which has become opaque while the protoplasm is still alive, is placed in normal saline the opacity disappears and normal development may continue; but as soon as it is replaced to tap water it dies. This really proves that it is possible to compensate for an injured capsule by rearing the egg in normal saline. The capsule, however, never returns to its normal condition as is shown when the egg is replaced to tap water.

Svetlov's vital staining experiments seem at first sight to prove his contentions. If eggs were immersed in neutral red or Nile blue sulphate the dye passed through the capsule and stained the contents intensely. Negative results followed similar experiments with trypan blue and Indian ink. Neutral red required 3 hours for the maximum effect, and Nile blue sulphate 12 hours. It is concluded that the egg capsule is permeable to some colloids under natural conditions. The first thing to notice is that neutral red and Nile blue sulphate belong to a toxic group of stains. Romeis [1928, p. 198] gives particular warning that even in highly dilute solutions neutral red is poisonous. Secondly, teleost embryos have been shown by Wislocki [1917] to be incapable of taking

up colloidal dyes in which they are immersed. Their impermeable integument distinguishes them from amphibia larvae [see McClure, 1918].

The writer has carried out vital staining experiments with the salmon (*Salmo salar*), the plaice (*Pleuronectes platessa*), and the goby (*Cyclogaster liparis*). Eggs were immersed in solutions of neutral red, methylene blue, ponceau S., and Janus green. Five hours' immersion in a 0·1 % solution of any of these stains coloured the capsule intensely, methylene blue being most readily adsorbed. When the shell was removed there was absolutely no trace of dye in the egg contents. Similar results followed three days' immersion in the dye. The perivitelline fluid of salmon eggs placed in a saturated solution of methylene blue for 15 hours was slightly stained, but the larvae within were either dead or dying. There was no case in which the stain passed through the shell and the embryo lived. The positive results only showed the staining reaction of dead tissue to dye-stuff.

A number of artificially hatched salmon larvae were left for 24 hours in dilute solutions of neutral red, methylene blue, trypan blue, and nigrosine. On examination they were found to be unstained. Using a 1 % solution the results were the same as long as the larvae lived, but when they were about to die a diffuse staining occurred. With plaice and goby larvae after hatching the results were the same. Thus Wislocki's observations have been entirely confirmed.

It is concluded that in Svetlov's experiments the dye injured the capsule before passing through, and injured the living tissue before staining it. In the absence of further evidence we may continue to believe that under natural conditions there is no exchange of electrolytic and colloidal substances through the shell.

The recent work of Straub [1929] presents the egg capsule in a new rôle. He analysed the causes of observed differences between the osmotic pressures in the yolk and white of hens' eggs. The vitelline membrane is permeable to both water and dissolved substances, nevertheless in the living egg the yolk keeps a higher osmotic pressure than the white for weeks. The difference is lost very quickly in an egg killed by cold. It requires a measurable expenditure of work on the part of the egg to maintain this condition. In general it is in the form of electricity that the free energy of a chemical reaction can be made to do work. The vitelline membrane must be expected to have the properties of a galvanic element, that is, some of the intermediate stages of glucose oxidation must take place in such a way that their free energy maintains a constant difference in potential across it. The membrane bears the same relation to the system as the copper wire between the poles bears to an electric battery. Neither is capable of acting alone as a producer of differences in potential. Straub predicts the histological structure that will be determined for the vitelline membrane of a hen's egg—a porous sheet in which the fluid-filled holes and solid parts provide the liquid and solid phases necessary for the passage of electric currents in both directions.

VARIATIONS IN WEIGHT AND THE RÔLE OF WATER IN DEVELOPMENT.

The data under discussion in this section are summarised in Tables I and II, and in Figs. 1 and 2. The curves are broken at hatching time owing to the loss of the shell and perivitelline fluid. Both egg and larval determinations have been made on the 65th and 70th days. The time over which hatching normally extends is shown on each graph by dotted ordinates, and broken lines give a similar indication of the approximate time during which the effects of hatching are felt. The special metabolic features associated with hatching show with particular clearness in the last eggs to hatch, and the changes in the curves caused by these eggs are of particular interest. As the larvae were selected from the main group at random the special features appear in varying degrees, so that before the effects of hatching are over some irregularity in the determinations occurs. In other words the internal factors—enzymes for example—which adversely affect the eggs during the last stages must injure the late-hatching larvae most.

Table I. *Variations in the dry material and water content during development.*

1 Days after fertilisation	2 Material	3 Wet weight	4 Dry weight	5 Water (%)	1 Days after fertilisation	2 Material	3 Wet weight	4 Dry weight	5 Water (%)
36	*e*	13·2784	4·3568	67·3	76	*l*	11·8804	3·9194	67·0
40	*e*	13·1162	4·2826	67·2	79	*l*	11·5903	3·7928	67·3
44	*e*	13·2764	4·2956	67·6	86	*l*	11·8374	3·7154	68·6
46	*s*	0·6750	0·2065	69·5	90	*l*	11·4210	3·4992	69·4
50	*e*	13·3762	4·3110	67·8	94	*l*	11·2256	3·4052	69·7
55	*e*	13·2046	4·3026	67·5	100	*l*	11·2000	3·2372	71·1
60	*e*	13·4595	4·3122	67·9	106	*l*	11·1556	3·0408	72·7
65	*e*	13·3143	4·2740	67·9	111	*l*	11·1808	2·9931	73·2
65	*l*	11·2842	4·0254	64·5	116	*l*	11·0876	2·8720	74·1
70	*e*	13·9796	4·2802	69·5	124	*l*	10·8034	2·5308	76·5
70	*l*	11·6766	3·8832	66·8					

Absolute figures represent the weights for 100 individuals in grams. In col. 2 *e* means eggs; *s*, shells; *l*, larvae.

Table II. *Growth of embryos and plastic efficiency coefficients.*

1 Days after fertilisation	2 Embryo wet weight	3 Yolk wet weight	4 Embryo dry weight	5 Yolk dry weight	6 Plastic efficiency coefficient	
					days	coeff.
36	0·223	12·450	0·036	4·115	—	—
86	1·113	10·724	0·175	3·543	—	—
90	1·612	9·809	0·261	3·235	86–90	0·413
94	1·784	9·442	0·288	3·116	90–94	0·094
100	2·698	8·502	0·432	2·804	94–100	0·462
106	3·744	7·414	0·598	2·445	100–106	0·462
111	4·076	7·105	0·652	2·342	106–111	0·524
116	4·630	6·458	0·741	2·132	111–116	0·424
124	6·035	4·768	0·962	1·573	116–124	0·396
—	—	—	—	—	36–124	0·364

Fig. 1 shows that there is no measured decrease in the dry weight until a few days before hatching. This is in line with the idea that there is no loss of substance through the shell. Just before hatching the membrane becomes thin

and loose, permitting an increased water content. The previously noted loss of fat in the egg capsule during development may partly account for the decrease in dry weight. In the last stages the capsule has become permeable and substance has passed through. Very little movement is possible for the larva within the egg, but after hatching it can swim about freely. The increased activity causes a more rapid loss in dry weight until an unexpected plateau appears at the 106th day, the former slope being resumed again 10 days later. May it not be that this period is one in which there is a change in the nature of the sources of energy with which the yolk provides the embryo for its building operations?

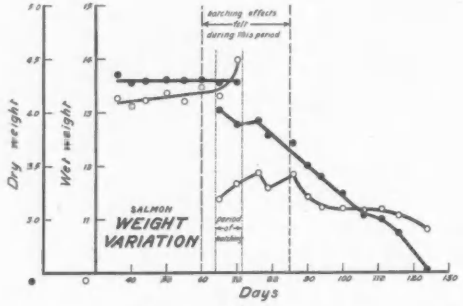

Fig. 1.

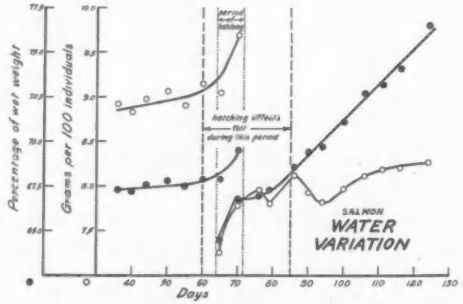

Fig. 2.

Studies on the mechanics of development of trout eggs and larvae have recently come from two sources. Since they differ somewhat with respect to observations as well as to theoretical conceptions it is necessary to examine them here with a view to determining which agrees more closely with related facts.

Kronfeld and Scheminzky [1926] measured the embryonic length and the wet and dry weights of the embryo, yolk sac, and egg capsule during development. The species used were *Trutta fario* and *Salmo lacustris* as well as hybrid eggs. In each experimental series they used eggs of one group with unfertilised

eggs as controls. The method involved preliminary hardening in 2 to 4 % formalin, but they produce evidence to show that this caused no significant changes. The water content of the egg as a whole did not appreciably vary up to the time of hatching. They have not distinguished between early and late hatching eggs and have therefore missed the sudden rise in the percentage of water which occurs just before the hatching of the latter. There was a loss of water at hatching, owing to the loss of the capsule and perivitelline fluid, followed by a rapid rise which continued until the end of development. They found that the water content of the embryo alone dropped until hatching and then rose suddenly, to reach its final level of 86 % three weeks later. The yolk sac alone contained 66 % of water in the early stages. From this tension the water dropped to as low as 50 % a week after hatching, followed by a rise so that the normal figure was reached again two weeks later. The developing embryo faced the problem of building living tissue of high water content from yolk of low water content. Presumably the living cells could only absorb water through the yolk sac, and for some days after hatching the water taken was not replaced. When the living tissue was almost in equilibrium again the water in the yolk sac commenced to rise. There was evidently some delicate adjustment of organism to environment, on the nature of which we may be able to speculate after considering the chemical changes [Hayes, 1930].

An investigation was also made of the specific growth rate during development, that is how much new tissue can be built per unit embryo per unit time at different ages. According to Kronfeld and Scheminzky's fig. 4 the curve of building power rose to two peaks, one of them 16 days before hatching, and the other 23 days after hatching. Anatomically the first maximum corresponded to the formation and growth of the chief organs of the body, the ensuing drop being due to a form of starvation owing to lack of water. The drop after the second maximum they attributed to the lack of some necessary material in the yolk, and they point out that this is the time at which hatchery experience has shown it is best to begin feeding the larvae.

Similar investigations to those just described have been carried out on the same species of trout (*Salmo fario* in British terminology) by Gray [1926, 1928]. The variations of the wet and dry weight of the egg or larva, embryo, and yolk (by difference) were found. The figures presented show that the embryonic water content remains constant at approximately 84 %, and that of the yolk at 59 %. These results indicate that the hatching process does not affect the water content of the yolk or embryonic body. The dry weights given in the second paper are calculated on this assumption.

Gray has also given figures for the specific growth rate during development, which are in agreement with those of Kronfeld and Scheminzky. His observations began at the 44th day, or after the time of the first maximum. The two curves inflect when the larva has completed about 70 % of its development. The agreement on this point may be accepted as proof that by this stage both the embryo and yolk have an unchanging water content. It is possible to

calculate the weight of the salmon embryo and yolk at this stage by weighing the whole larva wet and dry (see below). According to all workers the larval wet weight increases for some time after hatching, and then decreases until the embryo begins to take outside food. The water content however (Fig. 2), after the fluctuations subsequent to hatching, rises steadily until the embryo begins to feed. Therefore in the later stages the loss in dry material is greater than the gain in water. Increases in water are associated with the transformation of yolk into living tissue, and losses in dry substance are due to the catabolism of the embryo.

In Table II the plastic efficiency coefficients represent the ratio

$$\frac{\text{gain in embryonic dry material}}{\text{loss in yolk dry material}}.$$

This coefficient has been determined for a number of forms, and in a given species it may be assumed to be of the same order throughout larval development. It is given here for the salmon with the object of showing that by determinations of the wet and dry weight of the larva it can be decided independently whether the embryo and yolk have a constant water content. Data from the egg are invalid since two unknown variables are included, the shell and the perivitelline fluid. Assume, as Gray did, that both embryo and yolk actually maintain a stablised water content which may be found experimentally and applied to larvae of other ages. The dry weight of the embryo, yolk or larva is equal to the wet weight multiplied by the percentage of dry substance. The total larva is equal to the sum of the embryo and yolk. Instead of "yolk wet weight" can be written "larva wet weight minus embryo wet weight." Hence the following equation is true:

% dry wt. larva × wet wt. larva = % dry wt. yolk (wet wt. lar.– wet wt. emb.) + % dry wt. emb. × wet wt. emb.

The only unknown is the embryonic wet weight which is found by solving the equation. The equation can be conveniently expressed by a formula. Let T = total wet weight of larva given in col. 3, Table I; let x = total wet weight of embryo; let a = % dry weight of larva found by subtracting the value in col. 5, Table I from 100; let b = % dry weight of yolk experimentally found to be 33 in later larval stages; let c = % dry weight of embryo experimentally found to be 16 in later larval stages. The value 16 for c is among the highest found, but the position of any possible inaccuracy will be obvious when one remembers that it is dependent on the removal of all moisture from the outside of very small embryos dissected away from their yolk sacs. The fresh embryos used were fragile and it was difficult to remove all the water. The use of the figure 16 brings the theoretical results into harmony with direct observations. The following is the equation:

$$aT = b\,(T - x) + cx,$$

or it may be written

$$x = \frac{(b-a)\,T}{b-c}.$$

An attempt was made to apply this formula to each of the larval weights determined. The 65th and 70th day calculations gave the embryonic weight as a minus quantity; the 76th day as exactly zero; and on the 79th day 1·715 g. or double the experimentally observed value. The figures from the 86th day forward are given in col. 2, Table II, and in col. 6 the plastic efficiency coefficient (P.E.C.) is recorded. The latter figures (with one exception due to experimental error) agree quite well and show that after the 86th day the embryo and yolk have a constant proportion of water. The lack of uniformity of the calculations made before the 8th day provides independent evidence in favour of Kronfeld and Scheminzky's statement that the water content does not become stabilised for some time after hatching.

Col. 6, Table II also compares the P.E.C. over a long period (36 to 124 days) with that of the later larval stages. From this it may be seen that the coefficient increases somewhat during development. The 36-day figures quoted were obtained in the usual way and a correction applied for the shell. Their accuracy is probably not of a very high order, but this is offset by the small size of the embryo.

The form of the growth curves given by Gray, and Kronfeld and Scheminzky shows that after the second maximum an ever-increasing proportion of the yolk supplied is burned to provide energy of maintenance, while a decreasing share is built into new tissue. In other words basal metabolism steadily replaces growth. If no outside food were provided the embryo would finally cease to grow, become emaciated, and die. Thus if the P.E.C. given above had extended until the disappearance of the yolk sac it would necessarily have declined, indicating that the larva was wasting more food at the end of development. The conceptions of growth recently developed by Terroine and his collaborators show that this is not really the case. [Complete references may be found in Terroine and Wurmser, 1922, and in de Caro, 1928.] We are dealing here only with matter, and leaving out of consideration the energy of maintenance of the cells already formed. Any cell must carry on metabolism if it is to remain alive and this basal metabolism is not waste. The real energetic efficiency must be calculated according to the formula

$$\frac{\text{energy gained by embryo}}{\text{energy lost by yolk} - \text{energy of maintenance}}.$$

Some observers have attempted to distinguish between non-basal metabolism (wasteful burning of food) and basal metabolism (energy of maintenance) by measuring the surface area and assuming that the latter bears a constant relation to basal metabolism. Not wishing to rely on surface laws Terroine and Wurmser made their experiments directly, by finding the way in which the growth of slime moulds was hastened or retarded by adjustments of the p_H of the culture medium. The real developmental efficiency of different embryos, such as the frog, silkworm and chick, is of the same order. They waste from 20 to 30 % of the energy provided for their development. Accurate figures for the real energetic efficiency of a fish are not yet available.

SUMMARY.

1. The wet weight of a salmon egg increases slowly until a short time before hatching, then rapidly. In the larva the wet weight first increases and then decreases. The dry weight drops rapidly after hatching.

2. Observations on hatching show that the enzyme which dissolves the shell has a very unfavourable effect on the embryo.

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(*Note added to proof.*) In a further contribution (*Pflüger's Arch.* 1929, **223**, 645), Scheminzky discusses at length the discrepancies between his results and those of Gray. If Scheminzky discards his own experimentally obtained figures for the variations in embryonic water content during development, and assumes as Gray does that this is constant, he is able to apply Gray's equations to his other results and obtain a similar curve. He finds an agreement in experimental observation, the disharmony of the conclusions being due, first to the above mentioned assumption, and second to a difference in mathematical treatment. "Die Wachstumskurve nach *Gray* stellt somit einfach die in einem bestimmten Zeitpunkt vorhandene Menge von embryonaler Substanz dar, *ohne über die Intensität* des Wachstums eine Aussage zu machen."

LXXXIII. THE METABOLISM OF DEVELOPING SALMON EGGS.

II. CHEMICAL CHANGES DURING DEVELOPMENT.

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Introduction.

There is much evidence in the literature of embryonic metabolism which goes to show that during the course of its development the embryo makes use of foodstuffs as energy sources in the order carbohydrates, protein, fat. With this idea in mind Needham and Murray [see Needham, 1927] have recently ascertained the wet and dry weights, and the constitution, concentration, combustion, and absorption of yolk materials, of the developing embryo. The peak of carbohydrate combustion and absorption was found to be on or before the 4th day. With protein there was no correlation whatever between absorption and combustion; the peak of combustion at the 9th day came just at the trough of absorption, and by the time the peak of absorption was reached on the 16th day combustion had ceased to be a factor. A similar statement can be made for fat, the absorption of which rose to a peak by the 9th day, dropped to a trough by the 15th day and then rose until the end of development. Thus fat and protein complement each other during the later stages as sources of material for the formation of living tissue; their curves are reciprocals. It is probable that the cells of the blastodermal blood vessels which absorb the yolk and white into the embryo, have periods of varying permeability to water-soluble and fat-soluble substances. Of the end-products of protein combustion ammonia reaches a peak first, then urea, and finally uric acid. At the time of transference from protein to fat as a chief source of energy the cost of constructing 100 mg. of living tissue is greatest. The results of these workers are mentioned because they suggest the explanation to so many of the observations here recorded. It cannot be stated at present exactly to what extent the fish resembles the bird in the chemistry of its development. The scarcity of carbohydrate in fish eggs accounts for one difference.

Chemical analyses of Atlantic salmon (*Salmo salar*) eggs are not available but Greene [1921, 1, 2] removed mature ova from the body cavity of the king salmon (*Oncorhynchus tschawytscha*) and determined the following percentage composition: water 57·93, protein (alcohol precipitate) 26·79, neutral fat 10·42, phospholipins 2·0, organic extractives 1·42, glucose 0·09, inorganic ash

0·65. For trout eggs (*Trutta fario*) Fauré-Fremiet and Garrault [1922, 1, 2] have found the following percentage composition: water 58·5, protein (total nitrogen × 6·25) 29·81, neutral fats 4·20, phospholipins 3·46, cholesterol 0·58, carbohydrates 0·34, ash 1·25. They were unable to find glycogen in the egg, but determined that the reducing sugar isolated was glucose.

Some recent work, which will be further discussed, has led to the conception that an aquatic egg is essentially different from a terrestrial one in two ways. First the products of its protein combustion are excreted through the shell, and second the supply of water for the developing embryo is unlimited. The latter idea has been discussed at length in the foregoing paper [Hayes, 1930]. Protein metabolism is held to be limited in terrestrial eggs by some factor connected with the increasing concentration of nitrogenous end-products. Thus protein is a minor source of energy in terrestrial embryonic development, and a major source in aquatic embryos. Another explanation is possible, using the ever attractive theory of recapitulation. Up to about the 7th day the chick embryo develops like a fish. It combusts and absorbs chiefly protein. Then the vitelline membrane (corresponding to the fish egg shell) finally disappears and its place is taken by the serosa. The embryo gradually loses its dependence on protein, ceases to be a fish, changes into a bird. The fish at this stage hatches and continues to rely on protein as the main source of food supply during larval development.

Material and methods.

Eggs of the Atlantic salmon (*Salmo salar*) were procured in the eyed stage, reared, weighed, and dried as described in the first paper of this series. The dried material was then analysed for protein and fat.

Fat was extracted in a Soxhlet apparatus, the eggs having been previously pulverised; this latter procedure was unnecessary with the larvae. Extraction was allowed to proceed in the usual way for 3 hours with the siphon operating every 5 minutes, re-extraction of some of the samples having shown that this period was of sufficient duration to remove all the fatty constituents. After extraction the solvent was distilled off and the residue and extract dried to a constant weight. The extract was always slightly heavier than the residue loss. The former has provided the data recorded, except in one or two cases of obvious experimental error in which the residue loss, appropriately corrected, has been used.

The fat values given are the result of carbon tetrachloride extraction. The relative merits of ether and carbon tetrachloride as fat extractives have been discussed by several workers. Johnstone [1918] found 1·0 to 1·62 % of nitrogenous substances in carbon tetrachloride extracts of herring tissue. Bull [1928] preferred to use ether for similar work. It is always desirable, and particularly so in studies of developmental mechanics, to differentiate between lower nitrogenous substances such as protein building stones, aminolipins, etc., and the true proteins. Greene [1921, 1] calls the former substances "organic

extractives" to distinguish them from the neutral fats and phospholipins. In more general analyses they should be placed with the fats rather than with the proteins. Carbon tetrachloride is to be preferred as an extractive if it will remove these lower substances and thus permit a more accurate estimate of the proteins in the residue. If its extract contains slightly more nitrogen than ether extract of similar tissue it is fair to assume that this excess represents the simplest organic nitrogenous substances present. Differences in extractive power may be entirely due to temperature. In ordinary procedure ether extracts at slightly below its boiling point of 35°, while carbon tetrachloride extracts at slightly below 77°. Greene considered it necessary to modify his apparatus in order to ensure the highest possible temperature for extraction.

The first point was to determine whether there was a quantitative difference between the two types of extracts, and this was done using eggs of the common lumpsucker (*Cyclopterus lumpus*). Three samples were extracted with ether, then dried, weighed and extracted with carbon tetrachloride. The averaged results showed that 98·5 % of the total extract was removed by the ether and the remaining 1·5 % by the carbon tetrachloride. Estimations showed that the percentage dry weight of nitrogen in ether extracts is 0·287, and in carbon tetrachloride extracts it is 0·354. Combining these results we find that 19 % of the total nitrogen is contained in the carbon tetrachloride fraction. In other words the 1·5 % extra fat which the carbon tetrachloride removes contains 6·73 % nitrogen, a figure which is not far from the proportion in the egg as a whole. This extra nitrogen, however, is not capriciously taken from the main store of protein. Longer or repeated extraction will never remove any more, so that it must represent a specific type of compound in the egg—some product of protein breakdown. In a developing system such rearrangements are of the greatest interest, so that carbon tetrachloride, which differentiates to a greater extent than ether, is to be preferred as an extractive. It must be further noted that the nitrogen removed is in any case a relatively small fraction, amounting with carbon tetrachloride to 0·924 and with ether to 0·707 % of the total in the egg. Thus in about 25 % of the dried tissue extracted as fat considerably less than 1 % of the nitrogen is included. All these figures have been checked by another method, namely the analysis of eggs for nitrogen before and after extraction. The two sets of results showed close agreement.

Nitrogen was estimated by the Kjeldahl method, using 0·5 g. of dry fat-free substance. For percentage values it was necessary to apply a correction for the extracted fat. After some consideration it was decided to follow the classic practice of stating that the protein factor 6·25 is incorrect for fish tissues, pointing out why it is incorrect, and then using it. The question of non-protein nitrogen is discussed elsewhere.

The ash was determined by gently heating 0·5 g. of dry fat-free substance in a lightly covered silica crucible for 1 hour, then applying intense heat until the ash became colourless. Bruce [1924] has pointed out that some of the

volatile chlorides are driven off during the heating and some phosphorus in organic combination may be extracted with the fat, but these small quantities are within the limits of experimental error.

The Winkler method was used to determine the oxygen requirements of the developing eggs and larvae. A large receptacle was filled with tap water and allowed to stand until equilibrium with the atmosphere had been reached. Water was then siphoned from the receptacle into a 6-litre jar of accurately known capacity, from which samples were taken for oxygen estimation. One group of 1000 eggs was used for all the tests, these being placed in the jar and the lid sealed on, avoiding included air. Sometimes a bubble appeared in the jar during the course of the experiment, probably released from the slightly supersaturated water, but the error so introduced was not great enough seriously to affect the results. The duration of each test with eggs was 2 hours, a period in which less than 10 % of the available oxygen was used. The suddenly increased oxygen requirements after hatching necessitated a change in the routine, and 200 larvae were allowed to breathe for one hour in the jar. Burfield [1928] showed that the oxygen consumption rate of plaice eggs decreases in a closed vessel owing to the small quantities of carbon dioxide produced. Respiration was normal with a greatly diminished oxygen supply. The analyses here given indicate that the respiratory quotient is almost constant throughout development, so that by the standard methods adopted any decrease in oxygen consumption is the same in all cases and can be disregarded. During experiments the large jar was kept immersed in a tank to ensure standard temperature conditions. The eggs or larvae were kept frequently in motion by turning over the jar every 15 minutes. At the conclusion the lid was removed and water samples taken. A siphon reached to the bottom of the sampling bottle and water was allowed to flow over the top until three times its volume had passed through. Then the siphon was removed and the bottle stoppered without included air, so that the sample taken had not progressed toward equilibrium with the atmosphere. All determinations were made in duplicate, and care was taken frequently to standardise the sodium thiosulphate solution against potassium permanganate solution, which was itself frequently standardised against potassium tetra-oxalate. Infrequently one of the readings was wide of the mark and was discarded; in other cases the figures quoted are averaged from all four determinations.

Protein variations during development.

The results of the protein determinations are shown in col. 3, Table I, and in Fig. 1. The first important point is that the larva has a larger proportion of protein than the egg. The sudden rise at hatching can only mean that the protein is relatively more concentrated in the larva than it is in the shell and perivitelline fluid. But an analysis of egg capsules three weeks before hatching shows that the protein and water content are higher than in the egg as a whole. Some of the protein in the egg capsule has been destroyed before hatching by

the secreted enzyme. The rise in the total protein of the egg just before hatching is due to the method of calculation, which assumes that all the nitrogen is protein-nitrogen, which is never quite true and sometimes not as approximately true as at other times. Nitrogen is present in forms which are extracted with the fat (see next section). The measured protein content of the larva rises because yolk-protein of low nitrogen content is transformed into living tissue-protein of high nitrogen content. The sudden percentage decrease before hatching is merely a reflection of the greatly increased water content.

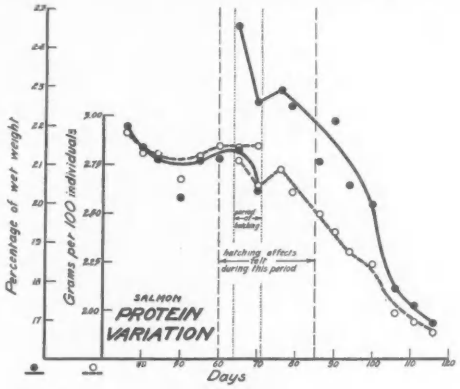

Fig. 1.

Table I. *Variations in the chemical composition and oxygen consumption during development.*

1 Days after fertilisation	2 Material	3 Protein %	4 Fat %	5 Remainder %	6 Smoothed remainder %	7 Oxygen consumption
36	*e*	21·98	3·98	5·49	5·45	0·060
40	*e*	21·42	4·94	5·19	5·31	0·075
44	*e*	21·17	4·81	5·17	5·17	0·068
46	*s*	22·30	—	—	—	—
50	*e*	20·15	4·93	5·87	4·97	0·066
55	*e*	21·20	5·01	3·04	4·80	0·055
60	*e*	21·15	5·00	4·70	4·63	0·052
65	*e*	21·43	4·94	4·48	4·46	0·049
65	*l*	24·54	3·00	6·71	5·49	—
70	*e*	20·35	4·47	4·63	4·29	—
70	*l*	22·60	4·20	5·15	5·16	0·433
76	*l*	22·90	4·06	4·79	4·77	—
79	*l*	22·50	4·06	4·89	4·58	0·471
86	*l*	21·05	5·83	3·17	4·12	—
90	*l*	22·10	5·63	1·62	3·86	0·729
94	*l*	20·45	4·87	3·73	3·59	0·629
100	*l*	19·96	5·16	2·53	3·20	0·956
106	*l*	17·80	5·41	2·94	2·82	—
111	*l*	17·36	5·06	3·13	2·49	—
116	*l*	16·91	5·18	2·54	2·18	—

Each oxygen figure is the requirement of 1000 individuals in 2 hours. Absolute figures represent the weights for 100 individuals in grams. For "remainder" calculations before hatching the averaged ash value of 0·16 g. or 1·25 % is used; after hatching 0·134 g. or 1·25 %. In col. 2 *e* means eggs; *s*, shells; *l*, larvae.

The activity of the free-swimming larva is shown by the rapid rate at which the protein is burned after hatching. The suddenly increased rate of combustion at the 100th day followed by a diminution 10 days later, marks this as a critical period in development. Morphological considerations offer no solution. Now Needham [1927] found that in the chick the point of maximum protein combustion is also the point at which the greatest quantity of substance is burned per unit of new tissue formed (this is not necessarily true on theoretical grounds), and it is just at this period that the transfer from fat to protein is going on. He cites Terroine, Trautman and Bonnet's results showing that such a transference results in an extra energy loss of 23 %. In the salmon fat begins to disappear at this point, and it also corresponds to the second growth rate maximum in the trout. A morphological examination shows that there is still an abundance of protein in the yolk sac. It is the physico-chemical properties of the organism and not a lack of food which determine why it should suddenly start to dilute its protein diet with fat.

The "remainder" values to be found in Table I and in Fig. 2, have been found by subtracting the total of the other values from the total weight of the egg or larva or from 100 (col. 5), and then smoothing these values into two straight lines corresponding to the periods before and after hatching (col. 6). (The values for total weight and water content are given by Hayes [1930], Table I.) The smoothed values were calculated using the simplest case of the method of moments where

$$y = a + bx$$

$$a = \frac{1}{2l} m_0$$

$$b = \frac{3}{l} \cdot \frac{1}{2l} \cdot \frac{m_1}{l}$$

y = vertical ordinate; x = distance from centre of range; $2l$ = range; m_0 = total area (sum of the frequencies); and m_1 = first moments about the centre of the range. The method is described by Elderton [1906]. The curves in Fig. 2 have been plotted along the smoothed values, although the points shown represent only the unsmoothed figures. (The ash content showed no significant variations during development. Its average for 100 eggs was 0·160 g.; for 100 larvae 0·134 g. The percentage throughout development was 1·25. These constants have been used to calculate the "remainder.")

Since the "remainder" values involve so many sources of error a high degree of conformity between the smoothed and unsmoothed values cannot be expected, but the points show clearly that at the time of hatching the slope of the curve suddenly steepens. The possible carbohydrate content of the eggs is small and cannot explain the "remainder." Water, fat and ash contents have been determined directly and any possible error could not vary with the developmental stage. With the protein, however, the procedure of multiplying the total nitrogen by 6·25 (or any other factor) involves the assumption that the

relation of nitrogen to protein is the same in different stages of development and in different egg constituents. From the entrance of the spermatozoon until the larva begins to take outside food there is nothing added to the egg except water, so that all the nitrogen must have been there from the beginning although more of it was missed in the earlier stages than later. Levene [1901] has shown that the nucleoproteins of the cod egg, which are especially rich in nitrogen, and moreover are required for the formation of cell nuclei, increase during development. The period of salmon development considered is one of rapid growth, and differences in constitution between embryo and yolk show with increasing clearness in successive analyses. Probably increasing nucleoprotein explains the form of the "remainder" curves. Stated otherwise the curves show that in the system as a whole the amount of protein corresponding to one unit of nitrogen is greater in the earlier stages than later, while the method of analysis assumes that it does not vary.

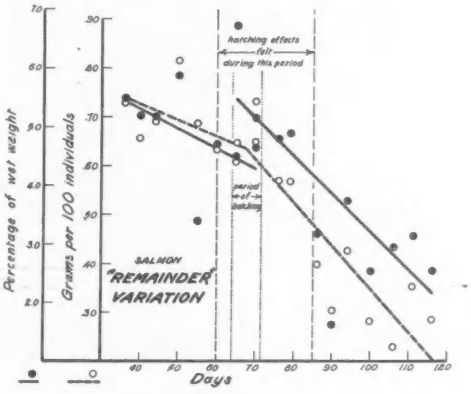

Fig. 2.

Since this difference between yolk and embryonic protein is a true one it is interesting to gain some idea of its magnitude. By what figure should the total nitrogen (after fat extraction) be multiplied in order to find the total embryonic or yolk protein? It is necessary to assume that substances other than water, protein, fat and ash are present in such small quantities that they may be disregarded. In col. 5, Table I, there is a "remainder" of 5·49 for the 36-day eggs. This means that on the above assumptions the correct protein value should be 27·47 instead of the 21·98 shown in col. 3. To obtain this value a factor of 7·81 must be used. From cols. 2 and 3, Table II [Hayes, 1930], the weight of the embryo and yolk at this age may be obtained and expressed as percentages; they are 1·76 and 98·24 respectively. If the same data are sought for the 116-day larva it is found that the larval protein factor necessary is 7·19, the embryonic weight 41·8, and the yolk weight 58·2. Let $x =$ the embryonic

protein factor, and let y = the yolk factor, and the following equations are true:

$$1{\cdot}76x + 98{\cdot}24y = 100 \times 7{\cdot}81$$
$$41{\cdot}8x + 58{\cdot}2y = 100 \times 7{\cdot}19.$$

The solution gives x a value of 6·36 and y a value of 7·82, which means that the embryonic protein contains 15·7 % nitrogen and the yolk-protein 12·8 %. Embryonic and yolk weights within the egg and other values could be obtained by the use of these factors, but such calculations are not justified without confirmatory evidence. Research in the choice of a factor in such investigations as this is obviously most desirable.

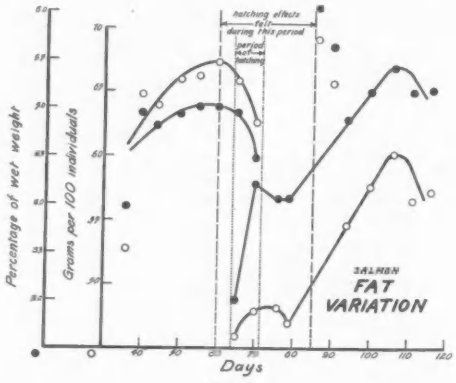

Fig. 3.

FAT VARIATIONS DURING DEVELOPMENT.

The fat extraction results are shown in col. 4, Table I, and in Fig. 3. The absolute and percentage values rise steadily until a few days before hatching, then decline suddenly. Hatching produces a further drop due to the loss of the shell and perivitelline fluid. During larval development the fat maximum is reached at the 106th day, following which both percentage and absolute values decrease. There is every indication that fat is being formed from protein during the early stages and again during the greater part of larval development. Since many fish eggs have very small quantities of fat their ability to synthesise it is a matter of very great importance in larval nutrition. A fat increase during development has been found in trout eggs by Tangl and Farkas [1904] and Gortner [1913], and in plaice eggs by Dakin and Dakin [1925]. With the plaice the amount of fat involved is exceedingly small, amounting to only 0·085 % in the beginning and 0·307 % at the end. Fig. 3 shows that there is a large decrease in late hatching eggs, owing to the necessity for the embryo to burn fat under the special conditions of hatching. Taken in relation to the protein the form of the fat curve suggests that there is one succession of energy sources in the egg and another in the larva.

Oxygen consumption during development.

The oxygen consumption records are given in col. 7, Table I, and the decrease in hatching is illustrated in Fig. 2. The figures serve plainly to emphasize what appears from all the other experiments; first that hatching produces a complete change in the conditions of development; and second that life processes drop to a low ebb under the unfavourable conditions previous to hatching. The great activity of the free-swimming larva causes its oxygen requirements to be about 10 times as great as in the egg stage.

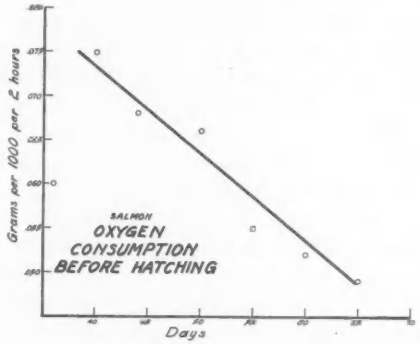

Fig. 4.

Kawajiri [1925] used the Winkler method to investigate the oxygen requirements of the Japanese land-locked salmon (*Oncorhynchus masou*). The variations in the egg stage were negligible, the curve showing a slight trough between the 5th and 10th days. A newly hatched larva consumed 5 times as much oxygen as an egg and the rate of absorption rose rapidly until the end of development. *Fundulus* eggs have also been studied by Scott and Kellicott [1916] and by Hyman [1921]. Their results agree except on one point, the decrease before hatching. Scott and Kellicott failed to find a decrease, but Hyman contradicts this result. Both found that at the time of gastrulation there is a marked rise while during the formation of the embryo there is no further increase. Another sudden rise when the heart begins to beat is followed, according to Hyman, by a gradual decrease until hatching. In the larval stages the oxygen requirements are greatly increased. Both workers followed the same method, namely the estimation of carbon dioxide by the p_H changes of the water in which the eggs were breathing.

The nature of growth.

In the later stages of egg development there is a gradual increase in water. It is important to know to what extent salmonid eggs are completely closed systems with respect to water, but there is some conflict in the data. In the salmon according to Rünnstrom [1920] and in the trout according to Svetlov [1929] there is a decrease in osmotic pressure during development, showing

that water is taken up. Gray [1920] found that in the trout the osmotic pressure of the yolk remains constant during development. Kronfeld and Scheminzky [1926] follow the conceptions of Tangl in regarding the egg as a completely closed system. Since the results given here agree with those of some other workers and have been obtained by direct measurement they may be accepted provisionally.

It had been believed that different osmotic pressures existed in the egg contents, but Svetlov [1929] found (on adequate theoretical grounds) that the embryonic osmotic pressure is the same as that of the yolk. Assuming that substances necessary for growth do not enter the egg from the environment, facts are now at hand for a revised story of the process of embryo building.

As the embryo grows the proportion of salt remains equal to that of the yolk, but that of the organic material is much lower. For building operations there must be a preferential selection of salt. For combustion on the other hand there is an exclusive selection of organic material. Within the egg little movement is possible and combustion amounts approximately to the minimum requirements for basal metabolism. It follows that if the water content of the yolk remained constant its proportion of salt would necessarily decrease. By some property, at the nature of which we can only guess, it maintains a constant salt content by expelling water into the perivitelline fluid which (as Svetlov showed) steadily increases during development. The decline after the first growth rate maximum shows that the increased concentration of colloidal material has changed the properties of the yolk. It cannot become indefinitely more concentrated—there is a plateau in the curve showing yolk water losses (Kronfeld and Scheminzky's fig. 2). In subsequent development the embryo becomes drier and takes more nearly equal proportions of salt and organic material. It also begins to burn fat. Speaking of the chick Needham [1927] states, "Out of 100 g. of protein in its diet the embryo can store away 98; out of 100 g. of carbohydrate 82; but out of 100 g. of fat only 43." If this relationship were true in the fish as well it would offer an explanation of the results. The embryo must maintain its level of water as constant as possible, and the mechanism adopted would be to use an energy source of which a large proportion was burned rather than built into new tissue. At the same time the enzyme which dissolves the shell is secreted and its unfavourable effects appear. At hatching the embryo is suffering from malnutrition, both embryo and yolk are lacking in water, but both have maintained their salt content. In the larval condition the embryo immediately begins to recover. It takes up water rapidly, and salt in proportion, so that the yolk loses water for a short time. The ultimate balance is restored by the greatly increased activity in the free-swimming state until finally the yolk has returned to its early water content level. This point has been shown to correspond to the second growth rate maximum in the trout (Kronfeld and Scheminzky). In the salmon the stabilisation of the water content comes just before the second change to fat as a source of energy. The embryo has grown so rapidly that the conditions of

the later egg stages have repeated themselves and the reaction is the same—the selection of fat as building material. There is a decrease in the growth rate and the larva first begins at this time to seek outside food.

These considerations bring the question of the succession of energy sources one step nearer to its solution. Attention is focussed on the salt content, and a fundamental question is to discover why it must remain constant and what happens when it is experimentally changed.

Summary.

1. In the developing salmon the protein decreases in the earlier egg stages and increases before hatching; in the larval stage it is burned rapidly. It has been calculated that embryonic protein contains 15·7 and yolk protein 12·8 % of nitrogen.

2. The fat content rises to two peaks, the first 10 days before hatching, and the second 6 weeks after. The decreases after the maxima are believed to show a change from protein to fat as a source of energy.

3. Variations in oxygen consumption indicate that metabolic processes drop to a low ebb before hatching and greatly increase in the free-swimming larva.

I am very grateful for the inestimable help of Prof. James Johnstone and Dr R. J. Daniel under whose direction this research has been carried out. Prof. J. M'Lean Thompson kindly permitted me to improvise hatching apparatus under especially favourable conditions in the Department of Botany, University of Liverpool. Much of the material used was provided through the courtesy of Mr J. G. D. Watts, Superintendent for the Dee Fishery Board. I also wish to thank the Nova Scotia I.O.D.E. and the International Education Board for financial assistance.

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LXXXIV. THE MICRO-DETERMINATION OF HYDROXYL GROUPS.

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Introduction.

During the course of other work, it was found necessary to estimate the hydroxyl groups in a substance believed to be a dihydroxy-alcohol [Marrian, 1929], of which only a small amount was available. A search of the literature failed to reveal any suitable micro-method. Experiments carried out on the hydrolysis of acetates of known purity led us to conclude that methods based on this were entirely unreliable when carried out on small quantities. The method based on the volumetric determination of the methane evolved when an alcohol reacts with Grignard's reagent as used by Tschugaeff [1902], Hibbert and Sudborough [1904] and Zerewitinoff [1907] seemed to be the most suitable for adaptation to a micro-scale.

Preliminary experiments made it quite clear that, unless certain precautions were most rigidly observed, entirely irregular results were obtained.

Hibbert and Sudborough emphasise the necessity of working in an atmosphere of nitrogen, since oxygen is absorbed by Grignard's reagent. Zerewitinoff, on the other hand, states that, since oxygen is only slowly absorbed by the reagent, it is unnecessary to work in an atmosphere of nitrogen. In our first experiments, when we did not observe the precaution of filling the apparatus with nitrogen, we were troubled with a rapid and prolonged contraction in volume of the gas in the burette, making it quite impossible to obtain a steady initial reading.

After blowing out the apparatus with dry nitrogen, this contraction was decreased greatly in magnitude, making it possible to obtain a steady initial reading after the lapse of some hours.

Very small variations in temperature and pressure should result in only a very slight error in macro-determinations when the volume of the gas burette is large relative to the total gas volume of the apparatus. In our micro-apparatus, the total gas volume was relatively enormous compared with the changes in volume which were being measured. Consequently small changes in temperature and pressure were observed to introduce appreciable errors. By suitable means the temperature was kept sufficiently constant, but variations in barometric pressure have constantly caused trouble. In unsettled weather, the pressure may change 3–4 mm. during the course of half an hour, making it

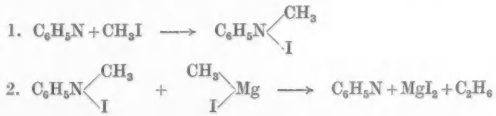
impossible to obtain steady readings. We found it necessary to observe the height of the barometer constantly during the course of an experiment, and if necessary to abandon the experiment for the day. The only alternative would be to reduce the total gas volume of the apparatus considerably. This would necessitate working with a much smaller reaction flask, which in many ways would be inconvenient.

The usual method of carrying out macro-determinations is to dissolve the alcohol which is being examined in some inert solvent, preferably the ether with which the Grignard reagent is made, and to allow this solution to react with the reagent. The difficulty of drying and keeping dry such a solvent suggested to us that for micro-determinations it would be preferable to allow the reagent to react with the solid alcohol. This method has been found to work excellently provided the alcohol is readily soluble in the ether of the Grignard reagent.

Preliminary determinations showed us that high results were invariably obtained unless the reaction flask and the substance being examined were dried with extreme care. Even so, blank determinations have always given an appreciable volume of gas. The magnitude of the blank seems to vary with the exact technique of cleaning and drying the flask, and also with the size and nature of the flask itself. With the same flask and with standard conditions of cleaning and drying, the blanks have been reasonably constant for a series of experiments. It has been our practice to do at least one blank determination to every two alcohol determinations.

A number of determinations on some of the common higher fatty alcohols were carried out satisfactorily using amyl ether and anisole Grignard reagents. Attempts were then made to estimate the hydroxyl groups in substances insoluble in these ethers or having only a very slight solubility. Determinations carried out on glucose and the dihydroxy-alcohol from the urine of pregnancy [Marrian, 1929] yielded the rather surprising result that no gas at all was evolved.

It was obviously necessary to dissolve such substances in some solvent that does not itself react with the reagent. Pyridine has been used by several workers in the past for this purpose, but there is some difference of opinion as to its suitability. Tanberg [1914], who was unable to obtain consistent results, criticises the use of pyridine. Zerewitinoff [1907] on the other hand claims that pyridine is well suited to the macro-method, giving consistent and accurate results if a few necessary precautions are observed in drying the pyridine and preparing the Grignard reagent. He suggests that the gas evolved when these precautions are not observed is ethane, produced according to the following equations:



and, therefore, if the Grignard reagent is quite free from methyl iodide, no gas is evolved.

With regard to the reaction between pyridine and Grignard's reagent we are in agreement with Zerewitinoff. When the reagent was prepared in the ordinary way, a large volume of gas was evolved when it was mixed with dry pyridine. If, however, the excess methyl iodide was first removed from the reagent by heating under reduced pressure, only a very small amount of gas was evolved with pyridine. The white solid compound that the reagent formed, even with dry pyridine, reacted completely but slowly with alcohols.

In spite of this the method proved to be a failure for micro-estimations, as the variations in the blanks caused by the introduction of another possible "wet factor" were so enormous. We thought at first that the method might be of use in distinguishing between the number of hydroxyl groups present in an alcohol, but even this is not too reliable, having an error up to 24 %.

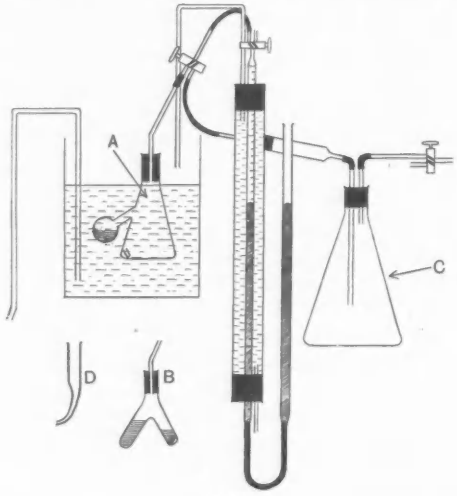


Fig. 1.

Experimental.

Apparatus.

The apparatus used was essentially the same as that previously described by Zerewitinoff (Fig. 1). The burette had a capacity of 5 cc., was 50 cm. long and graduated to hundredths of a cc. The flasks used were of two types (Fig 1 *A* and *B*). In all those experiments where pyridine was not employed, a flask of type *A* was used. Since, in the pyridine experiments, it was desirable to reduce the volume of the flask as much as possible to keep the blank to reasonable dimensions, a flask of type *B* was used having a capacity of about

one-half of that of type *A*. This second type of flask has been found to be very satisfactory and is probably preferable for all determinations.

The apparatus was kept as far as possible at a constant temperature by running a rapid stream of tap water through the jacket surrounding the burette, and through the water-bath in which the reaction flask was placed. In order to have as much as possible of the apparatus at a constant temperature, the rubber connections between the flask and the burette were made as short as possible.

A three-way tap connected the circuit with a large flask (*C*) which was kept filled with dry nitrogen, so that if for any unforeseen reason, *e.g.* change in barometric pressure, the mercury level in the burette was too high when the initial reading was to be taken, more nitrogen could be run into the burette.

Preparation of Grignard's reagent.

(*a*) *Amyl ether reagent.* The amyl ether was dried by standing over sodium for at least a week. It was then redistilled. 4·5 g. pure magnesium ribbon were then heated in a dry flask on a sand-bath with 25 g. methyl iodide, 140 cc. amyl ether and a small crystal of iodine under a reflux condenser for about 15 minutes to start the reaction. The flask was then removed from the sand-bath and the reaction allowed to proceed by itself for about one hour. When the reaction slowed down, the flask was again heated for about an hour to complete the reaction. In the case of the reagent used for the pyridine experiments, the flask was heated under reduced pressure for about 15 minutes to remove the last traces of methyl iodide. The reagent prepared in this way remained clear and active for 2–3 months if kept in a tightly stoppered flask filled with dry nitrogen.

(*b*) *Anisole reagent.* The anisole reagent was prepared by exactly the same method as described above, substituting anisole for amyl ether. This reagent was far less stable than the amyl ether reagent. In the course of two or three weeks it deposited a heavy white precipitate and lost its power of reacting with alcohols.

Drying and cleaning of apparatus.

The exact technique of cleaning and drying the reaction flask and the pipettes used for measuring the reagents has been found to affect the magnitude of the blank. As a minimum blank is essential the methods of cleaning and drying have been varied to some extent in the different series of estimations carried out.

In series *A* (Table I), the flask, which was made of ordinary glass, was washed out in succession with hot water, dilute hydrochloric acid, water and finally acetone. It was then dried for at least 3 hours in an oven at 110°. Before use the flask was cooled in a desiccator over calcium chloride for 30 minutes.

In series *B* the flask, which was made of silica, was cleaned first with hot water and then boiled in a solution of chromic acid for at least an hour. The

flask was then washed thoroughly with distilled water, heated to 110° for 30 minutes and finally to 170° for at least 3 hours over a dish of calcium chloride. As in series *A*, the flask was cooled for 30 minutes in a calcium chloride desiccator before use. The latter technique was adopted for series *C* (Table III) and series *D* (Table IV).

In each case the pipettes were cleaned and dried in the same way as the flask.

In preliminary experiments, we found that high results were invariably obtained unless special precautions were taken to ensure complete dryness of the substance under investigation. The bottle of substance was therefore left in a calcium chloride desiccator for several days before weighing the sample for the estimation. The sample, which was weighed into a small tube made by cutting down a hard-glass combustion tube to a length of about $\frac{1}{2}''$, was then placed in a desiccator over P_2O_5 for at least 24 hours before use.

Drying the pyridine. The pyridine was allowed to stand over anhydrous barium oxide for a week and then distilled into a bottle containing fresh barium oxide.

Method of determination.

The exact method of carrying out the determination with the solid alcohol (without pyridine) was as follows. For 30 min. nitrogen was passed first through a calcium chloride tube and then through the apparatus, entering at the nitrogen reservoir and leaving at the rubber connection to which the flask was to be attached. The mercury in the burette was then adjusted to a suitable level by raising or lowering the reservoir slowly. The flask was then taken from the desiccator and rapidly blown out with a stream of dry nitrogen. The tube containing the substance was dropped into the flask and 3 cc. of Grignard reagent run into the side tube through a pipette and the special funnel shown in Fig 1 (*D*). The flask was then fitted to the greased stopper previously attached to the rubber connection of the burette. This was invariably carried out in the late afternoon, and the apparatus left overnight before taking the initial readings, so that any residual oxygen could be completely absorbed. The following morning, readings of the level of the mercury were observed at intervals of 10 min. after the temperature of the bath had become constant. When at least four consecutive readings were the same, the flask was taken from the bath, the reagent tipped out of the side-tube on to the alcohol, and the flask shaken vigorously for some minutes. Alternate shakings and readings were made until the mercury level was constant. The flask was then replaced in the water-bath to attain the correct temperature and readings were taken every few minutes until constant, the whole operation usually taking about 15 min.

The procedure in the experiments where the alcohol was dissolved in pyridine was different in several details. After removing the flask from the desiccator and blowing it out with nitrogen, one of us (*A*) placed the tube containing the alcohol in one leg of the flask while the other (*B*) measured out

3 cc. of Grignard reagent. While *B* was running the reagent into the other leg of the flask, *A* measured out 1 cc. of pyridine and then ran it into the leg containing the solid alcohol. *B* then connected the flask to the burette.

The following morning we invariably found that a crust of white solid about 2 mm. thick had formed on the surface of the reagent. We suggest that this was due to the withdrawal of final traces of water from the pyridine.

On tipping the Grignard's reagent into the leg of the flask containing the pyridine solution of the alcohol, a precipitate, presumably an addition compound of pyridine and the reagent, at once formed. The thickness of this precipitate caused the evolution of the gas to be extremely slow, and shaking at intervals for at least 40 min. was found to be necessary for the completion of the reaction. By tapping the leg of the flask and then shaking, the curd could be broken and poured from one leg of the flask to the other. This hastened the evolution of gas considerably. The reaction was considered to be complete when three consecutive readings at intervals of 5 min. were the same.

Calculations and results

The volume of methane evolved in each experiment was corrected to 0° and 760 mm. From this the average corrected blank for the particular series was subtracted. The percentage hydroxyl in the compound could then be calculated from the formula:

$$\% \text{ OH} = \frac{17 \times 100 \times V}{22380 \times W},$$

where 17 is the molecular weight in g. of the hydroxyl group, V the corrected volume in cc., and W the weight in g. of substance taken. The results are shown in Tables I, II, III and IV.

Table I. *Amyl ether reagent.*

Blanks (at N.T.P.) 0·14 cc., 0·11 cc., 0·13 cc., 0·11 cc., 0·10 cc. Mean 0·12 cc.

Substance	Weight g.	cc. CH_4 at N.T.P.	cc. CH_4 corrected for blank	% OH Found	% OH Theoretical	% Error
Octadecyl alcohol	0·0113	1·04	0·92	6·18	6·29	−1·7
Octadecyl alcohol	0·0102	0·94	0·82	6·10	6·29	−3·0
Octadecyl alcohol	0·0102	0·96	0·84	6·25	6·29	−0·7
Octadecyl alcohol	0·0100	0·98	0·86	6·53	6·29	+3·8
Batyl alcohol	0·0123	1·75	1·63	10·06	10·88	−7·5
Batyl alcohol	0·0122	1·78	1·67	10·39	10·88	−4·5
Cholesterol	0·0098	0·67	0·55	4·27	4·40	−2·9

Table II. *Amyl ether reagent.*

Blanks (at N.T.P.) 0·09 cc., 0·08 cc., 0·05 cc., 0·07 cc., 0·09 cc. Mean 0·08 cc.

Substance	Weight g.	cc. CH_4 at N.T.P.	cc. CH_4 corrected for blank	% OH Found	% OH Theoretical	% Error
Octadecyl alcohol	0·0110	0·97	0·89	6·14	6·29	−2·4
Octadecyl alcohol	0·0101	0·91	0·83	6·24	6·29	−0·8
Hexadecyl alcohol	0·0108	1·06	0·98	6·89	7·08	−2·7
Hexadecyl alcohol	0·0102	1·03	0·95	7·08	7·08	0·0
Hexadecyl alcohol	0·0116	1·20	1·12	7·34	7·08	+3·6
Cholesterol	0·0110	0·68	0·60	4·15	4·40	−5·7
Cholesterol	0·0107	0·73	0·66	4·69	4·40	+6·6

Table III. *Anisole reagent.*

Blanks (at N.T.P.) 0·09 cc., 0·07 cc., 0·04 cc., 0·04 cc., 0·07 cc., 0·04 cc. Mean 0·06 cc.

Substance	Weight g.	cc. CH_4 at N.T.P.	cc. CH_4 corrected for blank	% OH Found	% OH Theoretical	% Error
Cholesterol	0·0105	0·64	0·58	4·19	4·40	−4·8
Cholesterol	0·0109	0·67	0·61	4·25	4·40	−3·4
Dilauryl alcohol	0·0106	0·72	0·66	4·73	5·00	−5·4
Dilauryl alcohol	0·0099	0·77	0·71	5·44	5·00	+8·8
Octadecyl alcohol	0·0092	0·78	0·72	5·94	6·29	−5·5

Table IV. *Amyl ether reagent. Substance dissolved in 1 cc. pyridine.*

Blanks (at N.T.P.) 0·40 cc., 0·17 cc., 0·38 cc., 0·25 cc., 0.27 cc., 0·09 cc., 0·22 cc., 0·26 cc. Mean 0·26 cc.

Substance	Weight g.	cc. CH_4 at N.T.P.	cc. CH_4 corrected for blank	% OH Found	% OH Theoretical	% Error
Cholesterol	0·0111	1·00	0·74	5·06	4·40	+15·0
Cholesterol	0·0082	0·81	0·55	5·09	4·40	+15·7
Cholesterol	0·0076	0·75	0·49	4·89	4·40	+11·1
Cholesterol	0·0096	0·69	0·42	3·32	4·40	−24·5
Hexadecyl alcohol	0·0082	0·89	0·63	5·83	7·03	−17·1
Hexadecyl alcohol	0·0110	1·16	0·90	6·21	7·03	−11·7
Hexadecyl alcohol	0·0092	0·99	0·73	6·02	7·03	−14·4
Quinol	0·0128	4·39	4·13	24·49	30·91	−20·7
Quinol	0·0026	1·16	0·90	26·27	30·91	−15·0

SUMMARY.

1. A micro-method for estimating the percentage of hydroxyl in the higher fatty alcohols, based on the macro-method of Hibbert and Sudborough [1904] is described.

2. Results accurate to $\pm 8\,\%$ are obtained when the alcohol is readily soluble in the ether of the Grignard reagent used.

3. The use of pyridine to dissolve alcohols which are not soluble in the ether of the reagent is not recommended, the results being very unreliable owing to a variable blank.

The authors wish to express their thanks to Prof. J. C. Drummond for his interest in this work. The expenses incurred were defrayed by a grant to one of us (G. F. M.) from the Medical Research Council, to whom we wish to express our gratitude.

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LXXXV. NATURAL ANTHOCYANIN PIGMENTS.

I. THE MAGENTA FLOWER PIGMENT OF *ANTIRRHINUM MAJUS*.

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(*Received May 1st, 1930.*)

From her study of the Mendelian factors for flower colour in *Antirrhinum majus*, Wheldale [1907, 1914] showed that, on crossing white with yellow or ivory varieties, plants bearing red or magenta flowers are produced. This led her to suggest that the red and magenta anthocyanin pigments might be derived from the yellow and ivory flavones, and that the white varieties, from which these pigments are absent, might carry the factors which act on the flavones to form anthocyanin.

With a view to testing this hypothesis, Wheldale [1913] commenced an investigation of the chemistry of these four pigments. With Bassett [1913, 1914, 1] she isolated the yellow and ivory flavone pigments and identified them as glucosides of luteolin and apigenin. The yellow flowers contained glucosides of both these pigments, while apigenin alone was present in the ivory variety. From the red and magenta flowers they isolated two amorphous pigments of high molecular weight and uncertain constitution, which were apparently uncombined with any acid [Wheldale and Bassett, 1914, 2].

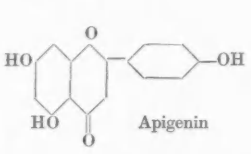
At this time Willstätter and Everest [1913] published an account of the isolation of the blue anthocyanin from *Centaurea cyanus* (cornflower). In this classical paper, they revealed the constitution and true nature of the anthocyanins, showing that they could be isolated by means of their stable crystalline oxonium salts. Unfortunately, this communication, which revolutionised the methods employed in the extraction and isolation of these pigments, was published too late to be of any use to Wheldale and Bassett, and the question of the relationship between these co-existing flavones and anthocyanins remained unanswered.

More recent research has still left the question open [Onslow, 1925] as to whether anthocyanins are derived directly from flavones or not: it is possible for instance that they are formed independently from the same parent substances.

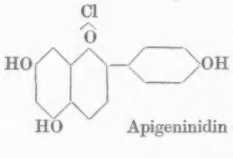
With a view to throwing more light upon the interrelationships between flavones and anthocyanins in the same varieties, a further attempt has been made to isolate and identify the red and magenta pigments of *Antirrhinum majus*. The pure crystalline chloride of the magenta pigment *antirrhinin* has been isolated by a process based on the methods of Willstätter and his co-workers. The preparation of the red pigment is to be attempted next.

If there were any evidence for the direct origin of anthocyanins from

flavones in the plant, the genetical relationships would point to apigenin as the parent substance of this magenta pigment. On crossing an ivory variety with a white of certain origin, a magenta offspring is produced. In this case the flavone apigenin is the only pigment present in either parent, and only this flavone and magenta anthocyanin are to be found in the offspring. If Wheldale's original hypothesis had been correct, some simple relationship should be apparent between the structures of these two pigments. Wheldale had originally suggested that the reaction occurring on the formation of anthocyanins would be one of oxidation. After the elucidation of the constitution of the anthocyanins, and the production by reduction, *in vitro*, of the anthocyanidin cyanidin from the flavone quercetin [Willstätter and Mallison, 1914], Everest [1918] supported the view that the process *in vivo* was one of reduction. If this second theory is correct, the magenta anthocyanin should prove to be apigeninidin, a pigment which has not yet been found to occur naturally.

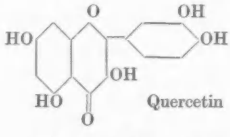


Apigenin

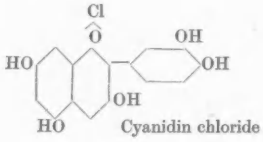


Apigeninidin chloride

The analysis of antirrhinin chloride, however, has shown it to be a 3-rhamnoglucoside of cyanidin chloride, which is closely related, structurally, to the flavonol quercetin and not to apigenin.



Quercetin



Cyanidin chloride

Since cyanidin contains one more molecule of oxygen than apigenin, the connection between these two pigments cannot be one of reduction, nor can one explain their relationship by any simple oxidative process. Wheldale and Bassett did not identify the sugar residues attached to the flavones of *Antirrhinum*, so that it is not yet known whether there is any correlation between the carbohydrate portions of these co-existing pigments.

The determination of the identity of antirrhinin therefore throws no further light upon the nature of a relationship, either of oxidation or reduction, between flavones and anthocyanins, which has been indicated by the study of the Mendelian factors.

In only four other cases have the constitutions of the co-existing flavones and anthocyanins been determined.

The red rose contains cyanin [Willstätter and Nolan, 1914], together with the corresponding flavone quercetin [Karrer and Schwarz, 1928], while the author (unpublished work) has recently identified a similar anthocyanin in

brown *Cheiranthus cheiri* (wallflower), from which quercetin and its monomethyl ether, *iso*rhamnetin, have already been isolated [Perkin and Hummel, 1896]. Thus in these two flowers the constitution of the pigments favours the theory of their interconversion *in vivo*.

The significance of the results in the case of *Viola tricolor* is at present not very clear, since Everest [1919] claims that myricetin is present in this flower together with the quercetin and delphinidin identified by Mandelin, and later by Perkin [1902] and by Willstätter and Weil [1916]. His claim, however, is not based on analytical results.

The flavone kaempferol [Perkin and Wilkinson, 1902] and the anthocyanin delphinin [Willstätter and Mieg, 1914] have been isolated from *Delphinium consolida*: thus here, as in *Antirrhinum*, no close relationship is apparent.

The information derived from the study of the constitution of these flavones and anthocyanins is therefore conflicting. There is no conclusive evidence that the plant produces the latter from the former, or that they are both formed by separate but parallel syntheses from the same parent substance.

The flowers used for this investigation were those of the deep crimson varieties, *Purple King* (Carter), *Purple Queen* (Ryder), and *Giant Crimson* and *Dwarf Crimson* (Sutton). They all presumably contained the magenta anthocyanin together with luteolin and apigenin. The plants, which were grown on a plot of land kindly lent to me by Miss E. R. Sanders of Newnham College, were planted and tended by the staff of the Cambridge Botanic Gardens.

The isolation of the pigment was carried out by a modification of Willstätter and Zollinger's [1916] method for the preparation of keracyanin chloride from the cherry. Dilute methyl alcoholic hydrochloric acid was used for the first extraction, since glacial acetic acid extracts contained large amounts of flavone which formed a gel, and made filtration very difficult. Owing to the flavone present even in the alcoholic extracts, great difficulty was experienced at first in obtaining the chloride of the pigment in a pure enough state for crystallisation, but this was at length achieved.

Purification of the pigment by means of the crystalline picrate was not possible in this case, owing to the extreme solubility of this salt. Use was therefore made of the insoluble lead salt. This was precipitated from the crude alcoholic extracts, and then dissolved in glacial acetic acid, in which the lead salts of flavones and other contaminating substances are not soluble.

The yield of pure pigment was small in proportion to the large amount of material used, but, as flowers were not lacking, it was found to be more economical in labour and reagents to start with large quantities, and subsequently to discard mother-liquors which still retained appreciable amounts of pigment.

The pure chloride of antirrhinin was finally isolated in four hydrated forms. These differed in colour and water of crystallisation, and each of them possessed a characteristic crystalline form.

The chloride of the sugar-free pigment was identified as cyanidin

chloride by means of its appearance, general properties and reactions, combustion analyses and products of alkali fission. In all these respects, perfect agreement was found with the account of this pigment given by Willstätter and Everest [1913].

It has also been possible to compare the pigment from *Antirrhinum* with a sample of pure synthetic cyanidin chloride which was very kindly sent to me by Prof. R. Robinson. They showed a close resemblance both in their absorption spectra and behaviour in a range of buffered solutions. This latter method, which has been introduced by Robertson and Robinson [1929], is an excellent and reliable means of comparing and characterising anthocyanins and anthocyanidins.

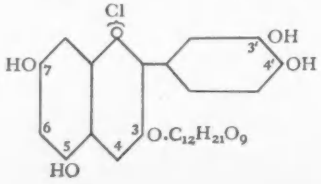
The identification of the sugar residue as a combination of a molecule each of rhamnose and glucose was determined by means of the distribution number, and qualitative and quantitative analyses of the sugars produced on hydrolysis.

The position of the sugar residue at position 3 in the antirrhinin molecule is indicated by the violet colour reaction given with sodium carbonate. If the substitution were at 5 or 7, the colour reaction would be either pure blue or violet blue, according to whether the hydroxyls at 7, 3, 3′ and 4′, or at 5, 3, 3′ and 4′, were unsubstituted. This decision is strengthened by the fact that, after hydrolysis of the glucoside, one obtains a pure blue reaction with the resulting cyanidin chloride from which the rhamno-glucosidal residue at position 3 has been removed. The validity of these colour reactions as a test for the position of the sugar residue has been studied by Robinson and his co-workers during their extensive syntheses, first of pyrylium compounds [Pratt and Robinson, 1925], and later of substituted anthocyanidins, and even of anthocyanins themselves [Robertson and Robinson, 1927, 1928].

Keracyanin, the pigment of the fruit of the cherry, not only has a similar constitution to that of antirrhinin, but appears from its reactions to be identical. Keracyanin chloride was isolated by Willstätter and Zollinger [1916] who describe two hydrates which resemble the red and brown hydrates of antirrhinin chloride.

The pigment isolated by Karrer and Widmer [1927] from the fruit of *Sambucus niger* (elderberry), and named by them sambucin, is also a rhamnoglucoside of cyanidin, probably together with traces of some other pigment. Many of the reactions of sambucin chloride resemble those of antirrhinin chloride, but no mention is made of the existence of different hydrated forms, and the crystalline picrate, which they isolated without any difficulty, must have been much less soluble than that of antirrhinin.

Antirrhinin chloride may be represented by the following formula:



Experimental.

The extraction and purification of the pigment.

The pigment was extracted with 2 % methyl alcoholic hydrochloric acid (8 litres) from about 10 kg. of fresh crimson flowers, from each of which both calyx and pistil had been removed. After standing for 2 days, the first extract was separated off, and the residue squeezed and re-extracted with a further 7 litres of acid-alcohol. Even after these two extractions some pigment was still retained.

A saturated aqueous solution of lead acetate was slowly added to the filtered extract until there was an excess. Lead chloride precipitated first; the solution then turned from red to purple, green, and then dark blue; finally the pigment was precipitated, together with the flavones, as a dark blue lead salt.

The mixture was allowed to stand for 2 days, by which time most of the pigment had been precipitated. After decantation of the red-brown mother-liquor, the precipitate was filtered, washed with a small amount of methyl alcohol, and sucked as dry as possible. A certain amount of pigment remained in the mother-liquor. Addition of ethyl alcohol precipitated some of this, and more came down on standing, but it was not possible to recover it all.

The moist, dark blue precipitate from two litres of alcoholic extract was extracted with successive amounts of glacial acetic acid, about 300 cc. being used in all. The extraction was incomplete, even on frequent rubbing and stirring. This was probably due to the retention of the pigment by the lead chloride. After standing for some hours, the dark-red acetic acid extract was again filtered from further impurities which had settled out.

On the addition of two volumes of purified ether, the lead salt was precipitated in dark blue granules, which were filtered off, washed with ether, dried and ground. The filtrate, which was green in colour, retained much of the impurity.

The rest of the original lead precipitate was purified in the same manner, about 100 g. of nearly pure lead salt being obtained in all. In some cases the ether precipitated the salt as a gummy mass, which absorbed water from the air. It was partially dried by rubbing with absolute alcohol and filtering. This deliquescence was probably due to the impurities present. It was noted, later on, that precipitation of the impure chloride of the pigment with ether generally gave a dry powdery residue, which quickly became sticky, but as purification proceeded, the pigment obtained was much less deliquescent.

11·0 g. of the finely ground lead salt were dissolved in a minimum of 5–10 % hydrochloric acid in methyl alcohol, and the lead chloride was filtered off. (If left to stand, a gel was formed due to flavone impurity.) One-third the volume of propyl alcohol was next added and the solution left overnight; the impurities which came down were filtered off, and, on addition of purified ether, the pigment was recovered as a flocculent, scarlet precipitate. The purity of the bright red, gummy mass, obtained on filtering, was tested by hydrolysing

a small amount. The sugar-free pigment which separated was mostly amorphous, but a few crystals were found on examination under the microscope.

The glucoside was next dissolved in a few cc. of warm methyl alcohol, made 20 % acid with hydrochloric acid and left to stand overnight. The chloride of the pigment was precipitated as a dark brown amorphous, or microscopically crystalline, substance, and a sample now gave, on hydrolysis, homogeneous needle crystals of the non-glucosidal pigment.

The glucoside was filtered, washed quickly with 20 % methyl alcoholic acid and dried by suction. The yield was 1·9 g.

The mother-liquor still retained much of the pigment. Part of this was later isolated, but great difficulty was experienced, as the impurities still present kept the pigment from precipitating.

The glucoside was recrystallised twice by solution in warm methyl alcohol, and addition of one-fifth the volume of concentrated acid. On standing overnight, the precipitation of the pigment as golden brown crystals with a bronze reflex was almost complete.

1·4 g. of pure antirrhinin chloride were thus obtained, and this method of purification was repeated on the rest of the crude pigment.

The glucosidal pigment, antirrhinin chloride $C_{27}H_{31}O_{15}Cl$.

Antirrhinin chloride can be prepared in at least four different hydrated forms. The appearance, composition, method of preparation and characteristics are given in the accompanying table:

Hydrate	Appearance	Properties	Method of preparation
Yellow hydrate $C_{27}H_{31}O_{15}Cl$, $1\frac{1}{2}H_2O$	Orange-yellow ppt. of fine, pale yellow, ill-defined needles. (See Fig. 1). Yellow powder when dry, turning brick-red after several weeks	V. insol. in cold water In hot water dissolves and isomerises instantaneously Barely soluble in cold or hot HCl solution (except in 3 %) Fairly soluble in cold alcohol, more soluble hot Insol. in neutral amyl alcohol, but more soluble when acidified	Precipitated from warm methyl alcohol solutions on standing. Precipitation not complete
Fan hydrate $C_{27}H_{31}O_{15}Cl$, $2\frac{1}{2}H_2O$	Dark purple-brown ppt. of feathery clusters of crystals resembling Japanese fans. (See Fig. 2)	Soluble in cold and hot water, alcohol and aqueous hydrochloric acid	Precipitated from strong (20 %) aqueous HCl; never from acid-alcohol. Often contaminated by the brown hydrate
Red hydrate $C_{27}H_{31}O_{15}Cl$, $3\frac{1}{2}H_2O$	Scarlet flocculent ppt. of fine needles. Brick coloured powder when dry. (See Fig. 3)	S. soluble in cold water. More soluble on heating Soluble in alcohol and in aqueous hydrochloric acid	Precipitated from aqueous 5 % HCl. A mixture of the red and brown hydrates separates from 10 % acid
Brown hydrate $C_{27}H_{31}O_{15}Cl$, $4?H_2O$	Brown crystalline ppt. with bronze reflex, composed of pale grey-brown leaflets in clusters. (See Fig. 4) Marks paper with a purple streak	V. soluble in cold water and alcohol V. soluble in dil. HCl, but decreasingly sol. in higher concs. More soluble on heating	Precipitated from 10 % acid methyl alcoholic solutions on cooling and standing

Fig. 1. The yellow hydrate.

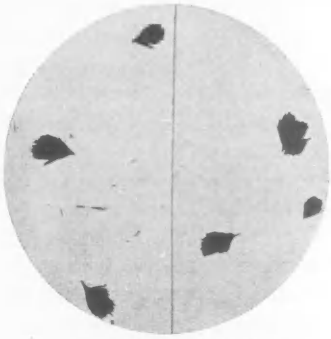


Fig. 2. The fan hydrate.

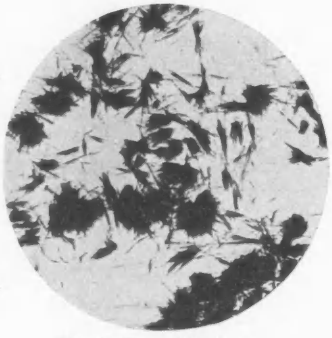


Fig. 3. The red hydrate.

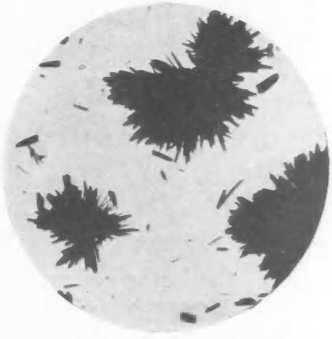


Fig. 4. The brown hydrate.

The following are the chief reactions of antirrhinin chloride:

Ferric chloride.	Fairly stable, good blue in alcohol. Violet, and less stable, in water.
Sodium acetate.	Violet colour.
Potassium acetate.	Plum-coloured solution.
Sodium carbonate.	Blue-violet to red-violet.
Sodium hydroxide.	Sapphire blue to green-blue.
Lead acetate.	Blue precipitate.
Perchloric acid.	Dissolves on warming. On standing, pale red-brown crystals of perchlorate separate in sheaves.
Picric acid.	Very soluble, no crystalline picrate separates.
7% *sulphuric acid.*	Soluble on heating. On standing, clusters of fine orange needles separate.
Fehling's solution.	Reduced.

The violet reaction with sodium carbonate indicates that the sugar-residue is attached to the molecule at position 3.

A solution of the colourless *pseudo*-base is easily produced on adding the pigment to a large volume of hot water, and heating. If acid is added to this solution, it reddens again, even in the cold. The purple colour base is partially precipitated when a concentrated water solution of the glucoside is heated.

A Zeisel estimation showed the complete absence of methoxyl groups.

The colour reactions and stability of antirrhinin chloride in a series of buffered solutions were determined by Robertson and Robinson's method.

The 17 buffer solutions, starting from p_H 3·2 and each increasing by 0·6 p_H, were prepared by the addition of suitable amounts of 0·2 *N* sodium hydroxide (CO_2-free) to 50 cc. of "Universal Buffer Solution" supplied by the British Drug Houses. The p_H values up to 11·0 were calculated by means of the formula supplied. Equal amounts (10 cc.) of each buffer were placed in a series of boiling-tubes, 1 cc. of an alcoholic solution of the pigment (25·0 mg. in 100 cc. 98 % alcohol) was added to each tube from a burette, and the tubes well shaken and examined, both at the moment of mixing, and at intervals during the next 48 hours. The results are given in the following table:

	p_H	Colour reaction and stability
10 % HCl		Salmon red
1 % HCl		Pink
I	3·2	Pink, fades quickly, but still pale pink after 3 days
II	3·8	Pink, fades less quickly
III	4·4	Pink, fades slowly
IV and V	5·0 and 5·6	Mauve pink, fades more slowly
VI	6·2	Mauve pink, more stable
VII	6·8	Brownish pink, fades slightly
VIII and IX	7·4 and 8·0	Brownish cherry, fades slightly
X to XII	8·6 to 9·8	Brownish cherry, very stable even after 16 hours
XIII and XIV	10·0 and 11·0	Bordeaux red, very stable even after 16 hours
XV		Cherry with blue tinge
XVI		Permanganate colour, slowly destroyed, pale brownish pink in 16½ hours
XVII		Deep violet, fades slowly
10 % NaOH solution		Pure blue, very unstable

The following figures were obtained for the loss in weight of the four hydrates, on removal of their water of crystallisation, by drying *in vacuo* over phosphorus pentoxide at 100° and 105°.

The 'yellow' hydrate

I. 77·8 mg. 'yellow' hydrate lost 1·8 mg. on drying at 100°	1·93 %
72·5 mg. 'yellow' hydrate (dried at 100°) lost 1·7 mg. on drying at 105°	2·35 %
Total loss in weight on drying at 105°	4·28 %
II. 179·1 mg. yellow hydrate lost 8·1 mg. on drying at 105°	4·52 %
Calculated for $C_{27}H_{31}O_{15}Cl$, $1\frac{1}{2}H_2O$	4·12 %

The 'fan' hydrate

271·4 mg. 'fan' hydrate lost 17·7 mg. on drying at 105°	6·52 %
4·837 mg. 'fan' hydrate lost 0·320 mg. on drying at 105°	6·5 %
Calculated for $C_{27}H_{31}O_{15}Cl$, $2\frac{1}{2}H_2O$	6·52 %

The 'red' hydrate

53·4 mg. 'red' hydrate lost 4·8 mg. on drying at 105°	9·0 %
34·0 mg. 'red' hydrate lost 3·0 mg. on drying at 105°	8·8 %
Calculated for $C_{27}H_{31}O_{15}Cl$, $3\frac{1}{2}H_2O$	9·0 %

The 'brown' hydrate

0·0790 g. 'brown' hydrate lost 0·0074 g. on drying	9·36 %
Calculated for $C_{27}H_{31}O_{15}Cl$, $4H_2O$	10·2 %

Micro-analyses of the substances after drying for carbon, hydrogen and chlorine gave the following figures:

4·821 mg. 'yellow' hydrate gave 8·960 mg. CO_2 and 2·18 mg. H_2O
5·20 mg. 'yellow' hydrate gave 1·09 mg. AgCl
4·517 mg. 'fan' hydrate gave 8·250 mg. CO_2 and 2·03 mg. H_2O
6·963 mg. 'fan' hydrate gave 1·623 mg. AgCl

The percentage compositions are therefore:

	C	H	Cl
'Yellow' hydrate	50·6	5·04	5·13
'Fan' hydrate	49·8	4·99	5·76
Calculated for $C_{27}H_{31}O_{15}Cl$ (cyanidin rhamnoglucoside)	51·39	4·92	5·62

The combustion analyses for the C and H content of antirrhinin chloride and the sugar-free pigment were carried out by Dr Ing. A. Schoeller of Berlin-Schmargendorf, and those for chlorine by A. Colwell of this laboratory.

The sugar-free pigment, cyanidin chloride, $C_{15}H_{11}O_6Cl$, H_2O.

The sugar-free pigment was obtained by hydrolysis of antirrhinin chloride with boiling 20 % hydrochloric acid for 4 minutes. After 2 minutes pale red-brown crystals began to separate in long needles (see Fig. 5). On cooling, filtering, washing with a little 20 % acid, and drying, a reddish chocolate-brown powder was obtained.

If dissolved in methyl alcohol, an equal volume of 10 % aqueous hydrochloric acid added, and left to evaporate, the pigment precipitates almost

Fig. 5. Cyanidin chloride from Antirrhinum.

quantitatively in well-formed crystals. These are not long and tapering as before, but are more sturdy and collect in stars and clusters.

When the pigment is dissolved in methyl alcohol, acid added and the alcohol boiled off, the salt reprecipitates in a curious symmetrical S-shaped form with curled offshoots.

The sugar-free pigment is slightly soluble in cold water, giving a mauve-red solution, which on heating turns purple, and then isomerises. It is difficultly soluble in aqueous hydrochloric acid of all strengths: on heating, it has a maximum solubility in 0·3 % acid, and in the cold in 0·03 % acid, a colour base being precipitated if this solution is heated. A hot saturated solution in 5·0 % acid does not contain much pigment, but on cooling even this is precipitated in fine needles.

The pigment is very soluble in methyl and ethyl alcohol, giving beautiful red-violet solutions which turn an intense cerise colour on acidification.

If water and not acid be added, a precipitate of the purple colour-base is immediately formed, and a green sheen appears on the sides of the tube. This behaviour is typical of cyanidin chloride.

Solutions in amyl alcohol are violet, and isomerise on standing. On acidification with an equal quantity of dilute acid, the alcohol layer turns crimson. On addition of alkali, the pigment passes into the water layer and turns blue. An acetone solution is violet.

Ethyl acetate dissolves the pigment to a small extent, giving a crimson solution on acidification, and a clear green-blue on addition of sodium hydroxide.

The chief reactions are:

Ferric chloride.	Blue colour in alcohol. Purple on addition of water. Unstable on addition of excess of reagent.
Sodium carbonate.	Violet and then pure blue.
Sodium hydroxide.	Clear green-blue, unstable.
Sodium acetate.	Red-purple solution in alcohol, purple in water.
Potassium acetate.	Dichroic red-blue in alcoholic solution. Colour base precipitated on standing.
Lead acetate.	Blue precipitate of the lead salt.
Perchloric acid.	Very soluble. No crystals of perchlorate are formed.
Picric acid.	Soluble on warming. Burr-like clusters of orange-red picrate crystals separate on standing.
7 % *sulphuric acid.*	Slightly soluble on heating. Brown hair-fine needles of sulphate separate on standing.
Fehling's solution.	Reduced slowly on heating.

It did not melt when heated up to 300°.

Acid amyl alcohol solutions gradually lose their colour on standing in the light. In a few days most of the pigment is destroyed.

A purple colour-base can be prepared by heating strong aqueous solutions of the pigment, from which it is quantitatively precipitated as an amorphous flocculent precipitate.

A solution of the colourless *pseudo*-base is obtained on adding an alcoholic solution of the pigment to a large volume of hot water. Addition of acid will not reproduce the red oxonium salt in the cold, but will do so on heating—a phenomenon peculiar to cyanidin chloride.

On fusion of the pigment with 50 % potassium hydroxide, small amounts of the breakdown products were isolated and found to be phloroglucinol and protocatechuic acid. The phloroglucinol was identified by means of its characteristic orange-red diazobenzene compound, the red colour given with a pine shaving and concentrated hydrochloric acid, the brick-red phloroglucide of methylfurfuraldehyde precipitated after heating with rhamnose and 20 % hydrochloric acid, and the darkening due to oxidation on addition of potassium hydroxide. The protocatechuic acid gave a green colour with ferric chloride followed by the very characteristic violet changing to violet-red on addition of weak sodium carbonate solution.

The crystalline chloride of the sugar-free pigment was dried at 105° *in vacuo* over phosphorus pentoxide, and eventually lost all its water of crystallisation. The last traces were very difficult to remove, and the whole amount was quickly absorbed again on standing in air. Combustion analyses were

therefore carried out on the crystalline and the dried pigment. The materials used in the third and fourth analyses were dried and undried samples of the same specimen. In the fourth analysis the agreement with the calculated values was better than that of the first three: a fact which points to the retention of a small proportion of water of crystallisation by the dried pigment.

On analysis, the following values were obtained:

0·1462 g. chloride dried at 105° lost 0·0082 g. = 5·58 %
0·1784 g. chloride dried at 105° lost 0·0092 g. = 5·16 %
$C_{15}H_{11}O_6Cl$, H_2O dried at 105° would lose 5·28 %

I (*a*). 4·854 mg. chloride dried at 105° gave 9·51 mg. CO_2 and 1·73 mg. H_2O
I (*b*). 5·904 mg. chloride dried at 105° gave 2·596 mg. AgCl
II. 4·009 mg. chloride dried at 105° gave 1·756 mg. AgCl
III. 4·682 mg. chloride dried at 105° gave 9·36 mg. CO_2 and 1·64 mg. H_2O
IV. 4·666 mg. chloride (air-dried) gave 8·995 mg. CO_2 and 1·70 mg. H_2O

The percentage compositions are therefore:

		C	H	Cl
By experiment.	Sample I	54·38	3·72	10·87
	Sample II	—	—	10·83
	Sample III	54·55	3·90	—
	Sample IV (air-dried)	52·57	4·05	—
Calculated for $C_{15}H_{11}O_6Cl$		55·81	3·42	11·00
Calculated for $C_{15}H_{11}O_6Cl$, H_2O		52·94	3·82	—

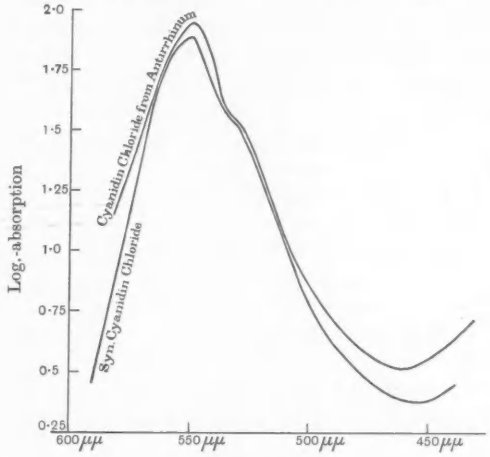


Fig. 6.

The sugar-free pigment was further identified as cyanidin chloride by means of its colour reactions, power of isomerisation and stability in the series of buffers. On comparing the pigment side by side with a sample of pure cyanidin chloride, the behaviour of these two pigments over the whole range was almost identical. Above p_H 8·6 the colour given by cyanidin chloride was slightly more violet, but this was only noticeable on very careful comparison. The reactions were similar to those described for cyanidin chloride by Robertson and Robinson.

The absorption spectrum of the chloride of the sugar-free *Antirrhinum* pigment was also compared with that of a synthetic sample of cyanidin chloride. From the readings taken with a spectrophotometer, the log. absorption curves were plotted over the visible spectrum (see Fig. 6), and were found to be very similar. The maximum absorption of the dark band in the green region is, in each case, at a wave-length of 550$\mu\mu$. Another band in the violet is also indicated. Acid (HCl) alcoholic solutions were used of such a strength that the maximum value found for the log. absorption lay between 1·75 and 2·0. Since the concentration of the synthetic cyanidin chloride solution was less than that of the *Antirrhinum* pigment, the curves, though parallel, were not identical.

The sugar residue.

The identification of the glucosidal residue of antirrhinin was carried out by means of the "distribution number," and by qualitative tests and quantitative estimations of the free reducing sugars and cyanidin chloride liberated on hydrolysis.

The "distribution number" for antirrhinin chloride was found by dissolving 0·005 g. of pigment in 25 cc. $\frac{0.5\%}{5\%}$ hydrochloric acid and shaking with two successive amounts of 25 cc. pyridine-free amyl alcohol. The amount of pigment extracted in each case was measured colorimetrically, by comparison with a standard made from 0·005 g. of pigment, dissolved in 1 cc. methyl alcohol, and made up to 25 cc. with amyl alcohol, a trace of acid being added to prevent isomerisation.

The mean value for the two extractions was 8·07, which compared well with the values for other rhamnoglucosides, which all lie between 6·5 and 10.

Qualitative sugar tests were carried out on the hydrolysis mixture, after filtering off the crystalline sugar-free pigment and removing any remaining traces of colour by extraction with amyl alcohol. A distinct smell of methylfurfuraldehyde could be detected during the hydrolysis. This is typical of rhamnoglucosides.

A portion of the filtrate was tested for pentose by the orcinol test. The solution turned pink (as for rhamnose) on boiling with orcinol and hydrochloric acid, and, on adding a drop of ferric chloride gave a green which was extracted by amyl alcohol. No blue precipitate (as for ordinary pentose) was obtained on boiling. The green colour was similar to that found with rhamnose, and was not of the vivid blue-green obtained with arabinose.

On hydrolysing for some time with strong hydrochloric acid, the distillate gave no pink with aniline acetate paper (therefore not normal pentose), but, on adding phloroglucinol, gave the characteristic yellow colour, and, on standing, a brick-red precipitate of the phloroglucide of methylfurfuraldehyde.

After neutralising the original filtrate with lead carbonate, filtering, and removing excess lead with hydrogen sulphide, the mixed osazone was prepared and compared with that from a mixture of pure rhamnose and glucose.

Quantitative estimations of the percentage of cyanidin chloride and reducing sugars obtained on hydrolysis of antirrhinin chloride were next carried out.

Weighed quantities of antirrhinin chloride (dried at 105°) were dissolved in 3 to 5 cc. of 1 % hydrochloric acid, and hydrolysed by boiling with 10 to 20 cc. 20 % acid for 2½ to 3 minutes. After standing in the ice-chest until the mother-liquor was barely pigmented, the crystalline cyanidin chloride was collected on a weighed Pregl glass filter, washed with a small amount of 20 % acid, dried *in vacuo* at 105° over P_2O_5, and weighed. One yield was air-dried, an allowance being made, in the calculation of the percentage, for the presence of one molecule of water.

After extraction of the remaining pigment with amyl alcohol, and subsequent removal of traces of the latter with pure ether, the filtrate was nearly neutralised with a small quantity of sodium hydroxide, and made up to a definite volume. The sugars were then estimated both polarimetrically and by Bertrand's method, it being assumed that rhamnose and glucose were present in equimolecular proportions. The polarimetric values obtained confirmed the identification of the hexose as glucose. Accurate polarimetric readings were taken by Mr B. Woolf of this laboratory, to whom I am much indebted.

The rhamnose content was estimated by the phloroglucide method [Ellett and Tollens, 1905], an aqueous solution of a weighed quantity of the glucoside being treated directly with 12 % aqueous hydrochloric acid.

I. 31·1 mg. antirrhinin chloride gave 16·80 mg. cyanidin chloride
31·1 mg. „ „ 13·59 mg. rhamnose + glucose (26·52 mg. Cu)
II. 187·7 mg. „ „ 104·20 mg. cyanidin chloride (air dried)
187·7 mg. „ „ 82·30 mg. rhamnose + glucose (161·7 mg. Cu)
III. 490·2 mg. „ „ 262·50 mg. rhamnose + glucose (50 cc., $l = 4$ dm., $\alpha_D = +0{\cdot}65°$)
IV. 135·8 mg. „ „ 65·02 mg. rhamnose + glucose (25 cc., $l = 2$ dm., $\alpha_{Hg} = +0{\cdot}19°$)
V. 231·4 mg. „ „ 62·40 mg. rhamnose (33·00 mg. phloroglucide)

The percentage yields are therefore:

	% cyanidin chloride	% combined sugars	% rhamnose
By experiment I	54·00	43·75	—
II	53·00	43·80	—
III	—	53·30	—
IV	—	47·80	—
V	—	—	27·00
Calculated for $C_{27}H_{31}O_{15}Cl$	51·11	54·57	28·86

An appreciable amount of sugar was broken down during the hydrolysis of the glucoside, the rhamnose being more easily destroyed than the glucose. The extent of this breakdown, which is shown in the low values obtained by both methods of analysis, is not so apparent in the polarimetric as in the copper reduction estimations, since, as the rotation of rhamnose (+ 9·0°) is small compared with that of glucose (+ 52·7°), a considerable loss of the pentose does not lower the combined rotation to a proportional extent. Willstätter found a similar discrepancy in the values obtained on estimating these sugars from keracyanin chloride and the flavone rutin by the same two methods.

SUMMARY.

1. The magenta anthocyanin, *antirrhinin*, has been isolated from the crimson flowers of *Antirrhinum majus* as a crystalline chloride.

2. Antirrhinin is shown to be a 3-rhamnoglucoside of cyanidin. It is probably identical with keracyanin, the pigment of the fruit of the cherry, but differs from sambucin from the elderberry.

3. The identification of antirrhinin as a cyanidin compound contradicts the theory of a simple relationship between this pigment and the ivory flavone apigenin, which is indicated by a study of the Mendelian factors for flower colour in *Antirrhinum*.

I should like to record my gratitude and thanks to the Hon. Mrs Wheldale Onslow for suggesting this piece of research, and for her help and advice; also to Sir F. G. Hopkins and Prof. R. Robinson for their interest in the work.

I also desire to thank the Department of Scientific and Industrial Research for a grant which has enabled me to complete these investigations.

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LXXXVI. NATURAL ANTHOCYANIN PIGMENTS.

II. THE MAGENTA FLOWER PIGMENT OF *PRIMULA POLYANTHUS*.

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(*Received May 1st, 1930.*)

Introduction.

In 1927 Karrer and Widmer [1927, 3] isolated and identified the anthocyanin pigments from three species of alpine *Primula*. They found that the flowers of *P. viscosa* and *P. integrifolia* both contained a pigment identical with malvin, a diglucoside of delphinidin-3′:5′-dimethyl ether, while from the flowers of *P. hirsuta* they isolated a diglucoside of a new *O*-trimethyldelphinidin, which they named hirsutidin. The pigment extracted from *P. integrifolia* was also shown to contain traces of some anthocyanin less highly methylated than malvin—a contamination to which these authors [1927, 1] had already drawn attention in the cases of other malvidin glucosides, such as oenin from grapes and myrtillin from bilberries.

Primulin, the anthocyanin pigment found in magenta and dark red flowers of *P. polyanthus*, is an interesting addition to this series. Like oenin, it consists chiefly of a 3-monoglucoside of malvidin, contaminated by traces of a lower methylated compound. Thus, although primulin furnishes the same aglucone as that found in two of Karrer's pigments, it differs from them in the nature and position of its glucosidal residue.

P. polyanthus is a horticultural hybrid probably derived from the cowslip and the common primrose. It is to be found in a large range of colours including various shades of yellow, magenta, crimson, orange and brick-red. Only magenta and crimson flowers were used for this investigation, as it is possible that a second anthocyanin is present in the orange and flame-coloured flowers.

The method of extraction and isolation was adapted from Anderson's [1923] modification of Willstätter and Zollinger's [1914] picrate method for the preparation of oenin chloride from purple grapes, in which 1 % aqueous hydrochloric acid instead of glacial acetic acid is used for the extraction of the pigment from the fresh flowers. Owing to the difficulty experienced in obtaining large quantities of material, only about 1 g. each of the pure crystalline hydrochlorides of the glucosidal and sugar-free pigments could be prepared.

The determination of the constitution of these pigments is based upon their appearance, reactions, solubilities, combustion analyses and absorption spectra, and upon the products obtained from the alkali fission and perhydrol oxidation of primulidin chloride.

The conclusion that primulin is not composed entirely of a monoglucoside

of malvidin is based firstly upon the low values obtained on estimating the methoxy-content of primulidin chloride, and secondly upon the transitory purple colour obtained with its alcoholic solutions on addition of ferric chloride. This colour, which is also given by oenidin chloride, must be due to the presence of small amounts of delphinidin, or some methylated delphinidin which does not contain the syringic acid nucleus, or of cyanidin, since it is only obtained with pyrylium salts possessing free hydroxyl groups at positions 3′ and 4′.

Since oenin, the pigment of purple grapes, also consists of a monoglucoside of malvidin, together with traces of a less methylated compound, a comparison has been made between the reactions of primulin and oenin chlorides, and of the chlorides of primulidin, oenidin and pure synthetic malvidin. Oenin chloride was prepared from purple grapes by a method similar to that used for the isolation of primulin chloride. The specimen of malvidin chloride, together with samples of three synthetic methylated malvidin compounds, was very kindly sent me by Prof. R. Robinson, to whom I am greatly indebted.

A comparison between the appearance, solubility and reactions of primulin and oenin chlorides shows that they resemble each other in most respects, but differ in their "distribution number."

Primulidin chloride resembles pure malvidin chloride in its sodium carbonate reaction and in its sparing solubility in hydrochloric and sulphuric acids. In crystalline form, difficulty of recrystallisation and ferric chloride reaction it resembles oenidin chloride. The absorption spectra of these three pigments are almost identical.

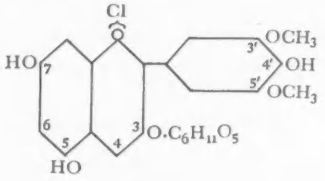
These anthocyanins and anthocyanidins were further compared by means of their colour reactions and stability in a series of buffered solutions [Robertson and Robinson, 1929]. Although primulin and oenin chlorides gave an almost similar range of colours, above p_H 6·8, those of primulin were slightly more blue. The chlorides of primulidin and oenidin differed in their behaviour slightly but definitely both from each other and from pure malvidin chloride. These comparisons further demonstrated the probability that, although both consist chiefly of malvidin monoglucoside, primulin and oenin are not identical, since they differ in the amount of contaminating pigment present.

On hydrolysis, primulin chloride yielded one molecule of primulidin chloride and one of glucose.

On addition of sodium carbonate, solutions of the glucosidal pigment gave a violet colour, while primulidin chloride gave a pure blue. This indicates that the glucose molecule is attached at position 3, thus preventing the production of the clear blue which is obtained with the sugar-free pigment which has a free hydroxyl group at position 3 as well as at 5, 7 and 4′ [Pratt and Robinson, 1925; Robertson and Robinson, 1927, 1928]. This position of the carbohydrate residue is further stressed by the fact that the colour given by primulin chloride on solution in a buffer of p_H 8·6 is identical with that given by the methylated malvidin compound synthesised by Bradley and Robinson [1928], in which the methyl group is at position 3.

Pure malvidin was first obtained as a crystalline chloride by Willstätter and Mieg [1914] from the wild mallow, in which it existed as the diglucoside malvin. Evidence that the methoxyl groups were in positions 3′ and 5′ was given later by Karrer and Widmer [1927, 1], who isolated syringic acid from the products of mild alkali fission, and decided to re-name the pigment *syringidin*. This structure was later confirmed by Bradley and Robinson's synthesis [1928] of a 3 : 5 : 7 : 4′-tetrahydroxy-3′ : 5′-dimethoxyflavylium chloride identical with malvidin chloride.

The constitution of 3-monoglucosidylmalvidin chloride, the chief constituent of primulin chloride, may therefore be represented by the formula below.



Experimental.

The extraction and purification of primulin chloride.

After the removal of their yellow centres and colourless corolla tubes, 600 g. of fresh red flowers of *Primula polyanthus* were placed in a large Kilner jar, 600 cc. aqueous 1 % hydrochloric acid added, the flowers pressed under the liquid and the air bubbles dispelled. The jar was loosely covered and left to stand for two days. The dark purple extract was then filtered. After grinding in a mortar, the residue was left to extract for two more days with a further quantity of acid (400 cc.).

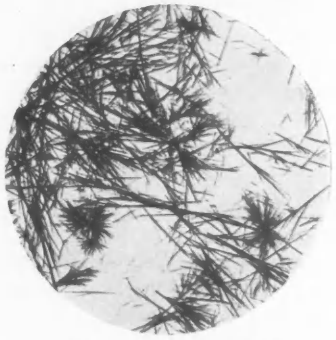


Fig. 1. Primulin picrate.

The first extract was warmed to 40°, and an equal volume of warm saturated picric acid added. The solution was kept at this temperature for a minute, and

then allowed to cool to room temperature. A flocculent precipitate of microscopic scarlet-red needles soon began to separate out. (See Fig. 1.)

After standing in the ice-chest overnight, the picrate was filtered off on a small Büchner funnel, washed free from picric acid with ether, and dried in a vacuum desiccator. As the red-orange filtrate still contained a considerable amount of pigment, it was saturated with picric acid and used to precipitate the anthocyanin from the second crude extract by the same method as before. After the separation of this second yield of picrate, an attempt was made to recover further quantities of pigment by making the extract 10 % acid with hydrochloric acid. More pigment was thereby precipitated, together with picric acid, but the bulk still remained in the mother-liquor and could not be utilised.

The two yields of picrate, together weighing 4·5 g., were very finely ground in a mortar, dissolved in 150 cc. methyl alcohol containing 7·5 cc. hydrochloric acid and filtered. On addition of about a litre of ether, most of the pigment was thrown down in dark red-brown granules with a bronze lustre. The mother-liquor not only retained the picric acid, but also some of the pigment, but the latter could not be fully precipitated even by the addition of light petroleum.

The chloride was ground finely, and washed with ether, until the washings no longer gave a yellow colour on addition of alkali. The yield of crude amorphous glucoside was 3·3 g.

To obtain the pigment in crystalline form, it was dissolved in a minimum quantity of an equal mixture of methyl and ethyl alcohol (about 10 cc.) and one-third the volume of 10 % aqueous hydrochloric acid added. On standing in a loosely covered vessel, purple rod-shaped crystals of primulin chloride separated out either singly or in aggregates. These were filtered off, washed with a little cold 10 % hydrochloric acid, sucked as dry as possible and air-dried at room temperature. They formed a purplish red powder which marked paper with a purple streak.

Part of this yield was recrystallised for analysis, the strongly coloured filtrate being used for the preparation of the sugar-free pigment.

The glucosidal pigment, primulin chloride, $C_{23}H_{25}O_{12}Cl$.

Primulin chloride is very soluble in cold water, giving a deep red solution. On dilution it turns a plum colour, and then isomerises, with no precipitation of the colour-base.

The pigment has a maximum solubility in aqueous hydrochloric acid between concentrations of 1·0 % and 2·0 %, but is less soluble as the concentration increases. In all concentrations, the pigment is dissolved to a greater extent on heating.

Primulin is very soluble in methyl alcohol, and nearly as much so in ethyl alcohol. Both give intense crimson solutions, which, on dilution with more alcohol, at once turn mauve, and are then decolorised through isomerisation.

Primulin is insoluble in ethyl acetate; only slightly soluble in acetone when neutral, but more so on acidification.

The chief reactions of primulin chloride are given below.

Ferric chloride.	Plum colour in alcoholic solution, changing at once to a stable orange-red.
Sodium carbonate.	Violet colour in alcohol. Blue in thin layers, red-violet in thick layers.
Sodium hydroxide.	Clear blue.
Sodium acetate.	Plum-coloured solution in water, and in alcohol. A purple colour-base (insoluble in benzene) is sometimes precipitated from strong solutions.
Lead acetate.	Blue-violet precipitate.
Fehling's solution.	Reduced.
Potassium acetate.	Blue precipitate.
Perchloric acid.	Very soluble, no crystals of perchlorate formed.
Picric acid.	Soluble on warming, scarlet-red needles of picrate separate on standing. (See Fig. 1.)
7 % *sulphuric acid.*	Soluble even in the cold, no crystals of sulphate separate.

On drying *in vacuo* over phosphorus pentoxide at 105°, primulin chloride loses all of its water of crystallisation, but quickly regains half of it on standing in air.

On analysis, the following figures were obtained:

I. 0·2441 g. primulin chloride lost 0·0375 g. on drying	15·37 %
II. 0·4850 g. primulin chloride lost 0·0754 g. on drying	15·60 %
Calculated for $C_{23}H_{25}O_{12}Cl$, $5H_2O$	14·55 %
Calculated for $C_{23}H_{25}O_{12}Cl$, $5\frac{1}{2}H_2O$	15·85 %

Primulin chloride therefore appears to possess $5\frac{1}{2}$ molecules of water of crystallisation.

I.	4·386 mg.	primulin chloride (dried) gave	8·390 mg.	CO_2 and 1·91 mg. H_2O
	6·189 mg.	,, ,, ,,	1·682 mg.	AgCl
II.	4·784 mg.	,, ,, ,,	9·100 mg.	CO_2 and 2·08 mg. H_2O
	5·422 mg.	,, ,, ,,	1·480 mg.	AgCl

The percentage compositions are therefore:

		C	H	Cl
By experiment.	Sample I	52·1	4·82	6·75
	Sample II	51·9	4·83	6·75
Calculated for $C_{23}H_{25}O_{12}Cl$ (oenin chloride)		52·2	4·74	6·74

The presence of methoxyl groups was demonstrated qualitatively by Zeisel's method.

The micro-analyses for carbon and hydrogen content of primulin chloride, and carbon, hydrogen and methoxyl content of the sugar-free pigment, were carried out by Dr Ing. Schoeller, of Berlin-Schmargendorf, and those for chlorine by A. Colwell, of this laboratory.

The colour reactions of primulin and oenin chlorides were compared in a range of buffered solutions, and found to be similar. Above p_H 6·8 the primulin chloride solutions were slightly more blue. The colours ranged from cherry-red at p_H 3·2, through rapidly fading violet-reds between p_H 3·6 and 5·6, and a fairly stable violet to violet-blue from p_H 6·8 to 8·0, to stable violet-blues (blue in thin layers) at higher p_H. In 10 % sodium hydroxide a very unstable blue was obtained. A violet colour-base was precipitated at all p_H values between 3·2 and 6·2.

Primulidin chloride, the sugar-free pigment, $C_{17}H_{15}O_7Cl$.

The crystalline sugar-free pigment, primulidin chloride, was obtained by hydrolysis of the glucoside with 20 % hydrochloric acid for 3 or 4 minutes. If heated too long, or if alcohol was present (as when the mother liquors from the crystallisation of primulin were hydrolysed), the pigment was partly amorphous.

Homogeneous crystals were prepared by dissolving the pure crystalline glucoside in hot 20 % hydrochloric acid and heating over a free flame for 4 minutes. The crystals began to appear after 2 minutes' boiling, and separated out almost quantitatively on cooling. Microscopically, they were pale brown or straw coloured rods, with blunt ends (see Fig. 2), their size depending upon the strength of acid used in the hydrolysis. The crystals were filtered, washed with a little cold 20 % hydrochloric acid and dried in air. They formed an almost black powder (pure malvidin chloride has a green reflex). The filtrate was later examined for the sugar residue.

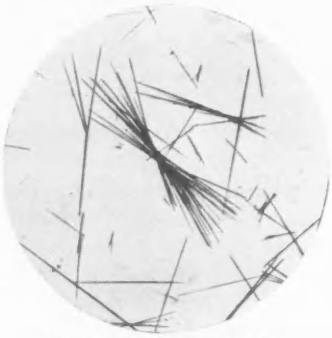


Fig. 2. Primulidin chloride.

Unlike pure malvidin chloride, primulidin chloride resembles oenidin chloride in being very difficult to recrystallise. Most attempts only gave amorphous forms, but small crystals were finally obtained by dissolving the pigment in water, and acidifying carefully until the solution contained 3 % of hydrochloric acid.

Primulidin chloride is slightly soluble in cold water, giving a red-brown solution after standing for half an hour. On heating, it is more soluble, but soon loses all its colour by isomerisation. The colour-base is precipitated from strong solutions.

The pigment is very soluble in methyl, ethyl and propyl alcohols, giving intense crimson solutions which, on dilution, turn purple and then isomerise. (Pure malvidin chloride is reprecipitated in small crystals from methyl alcoholic solutions.)

In amyl alcohol the pigment dissolves easily, giving a violet colour if neutral and cerise if acid. Like all anthocyanidins, on adding an excess of

aqueous alkali, the pigment leaves the alcohol, turns blue, and dissolves in the water layer.

Ethyl acetate dissolves the pigment to a small extent; while acetone does so fairly easily, forming a red solution when acid, violet when neutral, and a clear blue if made alkaline with sodium bicarbonate.

Though practically insoluble in either water or concentrated hydrochloric acid, the pigment is slightly more soluble in concentrations of acid between 0·005 % and 1·5 %. Maximum solubility in the cold is found at a concentration of 0·03 %, while, when hot, 0·3 % acid has the greatest solvent power. This pigment therefore resembles malvidin chloride rather than oenidin chloride in respect of its solubility in water and aqueous acid.

The following reactions are given by primulidin chloride.

Ferric chloride.	Purple in alcoholic solution. Very unstable. (Malvidin chloride gives no colour reaction.)
Sodium carbonate.	Violet colour and then pure blue.
Sodium hydroxide.	Blue colour, more green than with carbonate.
Sodium acetate.	Red-blue dichroic solution in alcohol, purple in water.
Potassium acetate.	Blue colour, followed by precipitation of a colour base insoluble in benzene.
Lead acetate.	Dark blue precipitate of lead salt.
Fehling's solution.	Vigorously reduced.
Perchloric acid.	Soluble on standing, aggregates of badly formed hair-like crystals of perchlorate are formed.
7 % *sulphuric acid.*	Fairly soluble, especially on heating; almost complete precipitation of fine needles on cooling (as for malvidin chloride).
Picric acid.	Soluble on warming, fine red hair-like crystals in clusters separate on cooling.

The substance did not melt on heating to 300°.

On drying in a vacuum at 105° over phosphorus pentoxide, the air-dried crystals lose all their water of crystallisation.

The following values were obtained on analysis:

I. 0·0742 g. primulidin chloride lost 0·0048 g. on drying 6·47 %
II. 0·2963 g. primulidin chloride lost 0·0187 g. on drying 6·32 %
Calculated for $C_{17}H_{15}O_7Cl$, $1\frac{1}{2}H_2O$ 6·87 %

I. 4·705 mg. primulidin chloride (dried) gave 9·16 mg. CO_2 and 1·9 mg. H_2O
4·260 mg. ,, ,, ,, 1·577 mg. AgCl
3·502 mg. ,, ,, ,, 3·20 mg. AgI
II. 4·721 mg. ,, ,, ,, 9·63 mg. CO_2 and 1·75 mg. H_2O
5·135 mg. ,, ,, ,, 1·863 mg. AgCl
3·195 mg. ,, ,, ,, 3·22 mg. AgI

The percentage compositions are therefore:

		C	H	Cl	OCH_3
By experiment.	Sample I	55·3	4·28	9·10	14·76
	Sample II	55·6	4·12	8·96	13·40
Calculated for $C_{17}H_{15}O_7Cl$ (malvidin chloride)		55·9	4·11	9·68	16·90

These figures are in agreement with the dimethyl formula $C_{17}H_{15}O_7Cl$, $1\frac{1}{2}H_2O$. The low values obtained for the (OCH_3) content indicate the presence of traces of a lower methylated delphinidin.

The colour reactions and stability of solutions of primulidin chloride were compared with those of oenidin and pure synthetic malvidin chloride in a series of buffered solutions. The different amount of contamination in oenidin and primulidin was demonstrated by the slight differences in the reactions of

Table I.

10 % HCl	p_H	Time of observation	Oenidin chloride (O)	Primulidin chloride (P)	Malvidin chloride (M)
I	3·2	Initial	Bluish red, stable	Same as O	Same as O
			Bluish red, fading	,,	,,
		30 min.	Very faint pink	Colour gone	Colour gone
II	3·6	Initial	A little bluer than I, fading	Same as O	Same as O
		30 min.	Very faint pink	Colour gone	Colour gone
III and IV	4·4 and 5·0	Initial	Same as II, but more intense	Same as O	Same as O, a little less intense
		30 min.	Very faint pink	All colour gone	All colour gone
V	5·6	Initial	Plum colour, more intense	Same as O	Same as O
		3½ hr.	Very pale pink	,,	,,
VI	6·2	Initial	A little more violet and more intense than V	Even more intense than O	Same as O
		3½ hr.	Very pale mauve	Same as O	,,
		18 hr.	Very pale blue	,,	,,
VII	6·8	Initial	Much bluer, pure blue in thin layers	Slightly more mauve than O	Same as O
		3½ hr.	Very pale blue	Same as O	,,
		18 hr.	,,	,,	,,
VIII	7·4	Initial	Same as VII	Slightly more mauve than O	Same as O
		3½ hr.	Paler and greener	Same as O	,,
		18 hr.	Very pale blue	,,	,,
IX, X and XI	8·0–9·2	Initial	Pure blue	Slightly more intense than O	Same as O
		3½ hr.	Paler and greener	Same as O	,,
		18 hr.	Very pale blue	,,	,,
XII	9·8	Initial	Same as XI	Slightly more intense than O	Same as O
		3½ hr.	Paler and greener	Same as O	Pale green-blue
		18 hr.	Dirty blue-grey	Dirty grey-yellow	Dirty grey-yellow
XIII	10·4	Initial	Slightly greener than XII	Pure blue as for XII	Same as P
		20 min.			Green-blue
		3½ hr.	Paler and greener	Same as O	,,
		18 hr.	Yellow (blue tinge)	Yellow	Yellow
XIV	11·0	Initial	Blue, greenish tinge	Slightly greener than O	Same as P
		20 min.	Pale blue	Pale blue	Pale green
		3½ hr.	Grey-blue	Grey-mauve	,,
		18 hr.	Yellow	Yellow	Yellow
XV		Initial	Same as XIV	Same as XIV	Same as P
		20 min.	Pale blue	Dirty blue	Pale green
		3½ hr.	Grey-blue	Yellow	Clear yellowish green
		18 hr.	Yellow	,,	Yellow
XVI		Initial	Same as XIV	Same as XIV	Same as P
		20 min.	Pale blue	Dirty blue	Pale green
		3½ hr.	Grey-blue	Yellow	Yellow
		18 hr.	Yellow	,,	,,
XVII		Initial	Same as XIV	Same as XIV	Same as P
		20 min.	Pale blue	Grey blue	Pale green
		¾ hr.	Grey-blue	Brown	,,
		3½ hr.	Yellow	Yellow	Yellow

the three chlorides, which are shown in Table I. It will be seen that below p_H 11 the primulidin chloride solution is slightly more mauve and more intense than the corresponding solutions of oenidin and malvidin chloride. At p_H 11 both primulidin and malvidin chloride are more mauve than oenidin, and at higher p_H these two pigments are destroyed in a shorter period of time. The resemblance between primulidin chloride and malvidin chloride appeared to be slightly greater than that between oenidin chloride and malvidin chloride. The buffered solutions were prepared by the addition of the increasing amounts of standard 0·2 *N* sodium hydroxide to the British Drug Houses "Universal Buffer Solution." Up to p_H 11·0, the p_H could be calculated accurately.

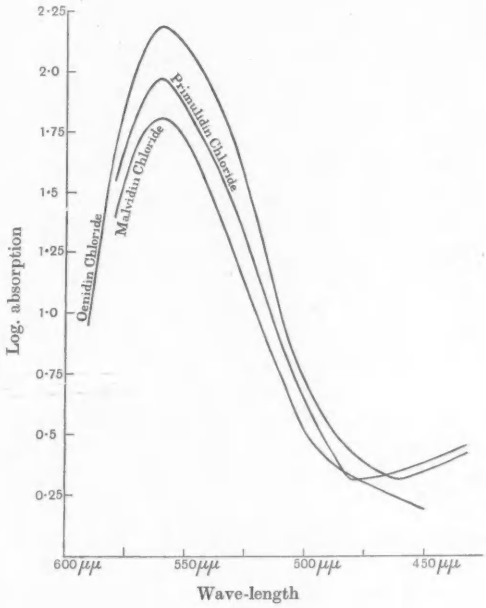


Fig. 3.

The absorption spectra of alcoholic hydrochloric acid solutions of oenidin, primulidin and malvidin chlorides were examined in the spectrophotometer, and the logarithmic curves were plotted to show the intensity of absorption of these three pigments within the visible spectrum. Each showed a band in the green portion, and a further one commencing in the violet and continuing into the ultra-violet region. The curves (see Fig. 3) were almost exactly alike, except at wave-lengths below 480$\mu\mu$ where the figures were unreliable, owing to the difficulty in taking accurate readings in the violet region. The maximum absorption in each case was at 560$\mu\mu$. The pigment solutions used were of such

a strength that the maximum log absorption values were at about 2·0. Owing to slight differences in these concentrations the curves obtained are parallel but not identical.

The breakdown of primulidin chloride by oxidation and alkaline hydrolysis.

After oxidation of primulidin chloride with hydrogen peroxide [Karrer and Widmer, 1927, 2], syringic acid was isolated from the decomposition products. About 0·2 g. of pigment was dissolved in 20 cc. hot water, and 15 cc. of 30 % hydrogen peroxide were added. After standing overnight, the brown residue was filtered off from the decolorised solution, which was then extracted several times with pure ether, and the ethereal solution washed free from peroxide by shaking twice with water. The ether was then evaporated off, and the pale brown residue was dissolved in a small amount of hot water, and decolorised by boiling with charcoal. On concentration of the solution in a vacuum desiccator, colourless needles separated. These were recrystallised twice, and identified as syringic acid by their melting point of 205° (uncorr.) and grey-green reaction with ferric chloride. The yield was very small.

A second sample of the pigment was treated for a few minutes with boiling 50 % potassium hydroxide, aerial oxidation being avoided. When hydrolysis was complete, the hot solution was poured cautiously into dilute sulphuric acid and extracted with ether. After treatment with sodium bicarbonate solution, through which carbon dioxide had been passed, it was necessary to extract repeatedly with ether to remove phloroglucinol. After evaporation of the ether, the pale yellow residue was taken up in water. The yield was very small, owing to the small amount of pigment available for the experiment, and the ease with which phloroglucinol is oxidised during the hydrolysis. It was possible, however, to identify it by the red colour obtained with a pine shaving and strong hydrochloric acid, by its orange-red diazobenzene compound, and by the precipitation of a brick-red phloroglucide on boiling with rhamnose and 20 % hydrochloric acid.

On acidification of the aqueous layer, extraction with ether, and evaporation, a pale brown residue was obtained, which appeared from its acid reaction and colour with ferric chloride, to be a mixture of gallic and syringic acids.

The sugar residue.

The identification of the carbohydrate portion of the primulin molecule was carried out by means of qualitative tests and quantitative estimations.

The "distribution number" for primulin chloride was ascertained by dissolving 0·005 g. in 25 cc. 0.5% hydrochloric acid, and shaking with two successive amounts of amyl alcohol (25 cc.). The amount of pigment extracted in each case was measured colorimetrically by comparison with a standard made from 0·005 g. of pigment, dissolved in 1 cc. of methyl alcohol and made up to

25 cc. with amyl alcohol. The amyl alcohol was pyridine-free and contained a trace of hydrochloric acid to prevent isomerisation.

The mean value obtained for the distribution number of primulin chloride was 8·6. This is somewhat below the values usually found for monoglucosides (9·8 to 11·0), but is considerably above those for diglucosides, which are all below 2·0. (Oenin chloride has a distribution number of 10·4.)

About 0·1 g. of glucoside was hydrolysed with 10 cc. of 20 % hydrochloric acid. After cooling, the sugar-free pigment was filtered off, and a small amount of colour which still remained in solution was removed by shaking with amyl alcohol. The absence of pentose from the filtrate was shown by a negative orcinol test.

The remaining sugar solution was shaken with ether to remove any amyl alcohol remaining and neutralised with lead carbonate. After filtering, the excess lead was removed with hydrogen sulphide, and the latter was expelled from the filtered solution by heating on a water-bath.

The osazone was prepared from this neutral solution in the usual way. It resembled glucosazone in crystal form and in its melting point of 210° (uncorr.), which did not change after three recrystallisations from methyl alcohol.

The amount of glucose in the primulin molecule was estimated by determining the proportions of primulidin chloride and reducing sugar produced on hydrolysis of the glucoside.

A weighed quantity of primulin chloride (dried at 105° as before) was hydrolysed with 10 cc. of acid and left in the ice-chest until the mother-liquor was barely pigmented. The crystalline sugar-free pigment was collected on a weighed Pregl glass filter, washed with a small amount of 30 % hydrochloric acid, dried *in vacuo* at 105° over P_2O_5 and weighed.

The filtrate was extracted with amyl alcohol, shaken with pure ether to remove any remaining alcohol, neutralised with sodium hydroxide and made up to a definite volume. The reducing sugar present was estimated by Bertrand's method and polarimetrically.

I. 128·9 mg. primulin chloride (dry) gave 88·8 mg. primulidin chloride (dry)
128·9 mg. „ „ „ reduced 80·0 mg. copper≡39·82 mg. glucose
II. 84·1 mg. „ „ „ gave 23·7 mg. glucose (25 cc., $l=2$ dm., $\alpha_D=+0{\cdot}10°$)
III. 53·9 mg. „ „ „ gave 36·5 mg. primulidin chloride (dry)

The percentage yields are therefore:

	% $C_{17}H_{15}O_7Cl$	% $C_6H_{12}O_6$
By experiment I	68·8	30·9
II	—	28·2
III	67·7	—
Calculated for $C_{23}H_{25}O_{12}Cl$	69·4	34·1

The low sugar values obtained are due to the breakdown of an appreciable amount of glucose during the hydrolysis.

The analyses for the carbon, hydrogen and chlorine content of primulin chloride are also in agreement with the monoglucosidal formula. The polarimeter reading is correct for glucose, and not galactose.

The colour reactions of primulin, primulidin, oenin, oenidin and malvidin chlorides, and of three synthetic malvidin compounds methylated at 3, 5 and 7 respectively, were compared in buffered solutions of p_H 8. It was found that the chlorides of primulin, oenin and 3-*O*-methylmalvidin gave a similar violet colour (violet-blue in thin layers, and red-violet in thick layers). On the other hand, primulidin, oenidin and malvidin chlorides gave a similar pure blue, 5-*O*-methylmalvidin chloride gave a slightly less green-blue, and the 7-methyl compound gave a violet-blue (pure blue in thin layers, and blue-violet in thick layers), which was easily distinguishable from the violet colour given by the 3-substituted compounds. It is therefore reasonable to suppose that the glucose molecule in primulin is attached at position 3.

Summary.

1. The magenta and dark red flowers of *Primula polyanthus* contain an anthocyanin pigment which has been named *primulin*.

2. By extraction of the pigment with dilute hydrochloric acid and purification through the picrate, pure crystalline primulin chloride has been prepared, and shown to consist of 3-monoglucosidylmalvidin chloride, together with traces of a lower methylated compound.

3. By a comparison of the properties and reactions of the chlorides of primulin and oenin and of the sugar-free pigments primulidin, oenidin and malvidin, it is shown that, although of nearly the same constitution, primulin and oenin are not identical.

4. Primulin furnishes the same aglucone as the pigments of *P. viscosa* and *P. integrifolia*, but differs from them in the nature and position of attachment of its glucosidal residue.

My thanks are due to the Hon. Mrs Wheldale Onslow for her help and advice, to Prof. R. Robinson for criticisms and suggestions and to Sir F. G. Hopkins for his interest in the work.

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LXXXVII. THE INFLUENCE OF INSULIN ON THE DISTRIBUTION OF GLYCOGEN IN NORMAL ANIMALS.

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Since the action of insulin began to be investigated, a puzzling discrepancy between its action on the diabetic and on the normal animal has attracted attention. In the diabetic animal it readily renews the store of glycogen in the depleted liver; on the other hand, the predominant result of the earlier and of much of the later work was to show that, when injected into the normal animal, if it measurably affected the glycogen in the liver or the muscles, it caused disappearance instead of additional accumulation [cf. Dudley and Marrian, 1923; Macleod and McCormick, 1923; Babkin, 1923; Nitzescu and Popescu-Inotesti, 1923; Brugsch, 1924; Gigon and Staub, 1923]. There was evidence, indeed, that insulin even in the normal animal might initially promote an increase of glycogen in the liver of the rabbit [Cori, Cori and Pucher, 1923], or of the whole mouse [Bissinger, Lesser and Zipf, 1923], though at a later stage of developed hypoglycaemia the glycogen became reduced below the original amount. Cori later found that insulin caused diminution of liver-glycogen in rabbits, guinea-pigs and mice when the proportion was initially high, but not if it was low. There was no room for doubt, however, that the glycogen stores of liver and muscles might in some species be seriously depleted by insulin with a concurrent great reduction of oxidative metabolism; and the problem remained of accounting for the disappearance of circulating glucose under such conditions.

Macleod [1926] and his school have interpreted such results as pointing to the conversion of glucose into some substance unrecognisable by the ordinary methods of estimating carbohydrates, when insulin acts on the normal animal. It was found in this laboratory, however, that when insulin acted under uncomplicated conditions on muscle, the glucose disappearing could be fully accounted for by glycogen storage and oxidation [Best, Dale, Hoet and Marks, 1926]. It was accordingly urged that the phenomena seen in the whole animal were due to complication by another action of insulin, namely, a depression of the new formation of carbohydrate, so that oxidative metabolism was concentrated on that pre-existing, with consequent depletion of reserves.

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Substantially similar views as to this effective transfer of oxidation to carbohydrate had been put forward by Laufberger and by Cori and Cori.

More recent evidence has suggested yet another factor in the total effect. Cori and Cori [1928] have shown that in the rat a suitable dose of insulin diminishes the store of glycogen in the liver and increases that in the muscles, while a small dose of adrenaline affects the distribution in the other direction, causing glycogen to leave the muscles as lactic acid, which the liver rebuilds into glycogen. Experiments by Marks and myself, which are being published elsewhere, have confirmed this effect of adrenaline on muscle-glycogen and brought to light a new discrepancy, since we failed to find enough lactic acid to account for the glycogen thus discharged. Since a hypoglycaemic dose of insulin provokes accelerated secretion of adrenaline, the bearing of this complication on the total effect of an insulin injection had to be considered, and gave promise of reconciliation between the rival conceptions above mentioned.

Meanwhile, however, Goldblatt [1929] had published the results of experiments, made chiefly on young, fasting, normal rabbits. He found that injection into such animals of a dose of insulin sufficient to cause a profound hypoglycaemia, without convulsions, produced a substantial accumulation of glycogen in the liver. The change shown in his experimental records was striking, especially in view of the fact that so many workers, under conditions but slightly different, had looked for such an effect and failed to find it, or had even observed its direct opposite. It proved to be easy, as I shall show, under the conditions of Goldblatt's experiments, to confirm his observation with regularity. He has based on it a theory that the action of insulin is simply to retard the conversion of liver-glycogen into glucose, with resulting accumulation of glycogen and hypoglycaemia.

Such a theory, attractive in its simplicity, might have been easy to accept at a very early stage of the investigations on insulin. Its acceptance to-day would appear to involve the rejection of a large body of evidence obtained by other workers, on a number of species and by various methods. It seemed desirable to examine further the relation of the fact observed by Goldblatt to many which appeared to conflict with it. With this object in view I have studied the effects of nonconvulsant doses of insulin in normal animals of several species.

Experimental.

Methods.

In all experiments convulsions were avoided, the animals being killed when preconvulsive flaccidity and inco-ordination had developed. The animals were killed either by a blow on the head or, in the case of mice and fowls, by decapitation. The thorax and abdomen were quickly opened and blood for sugar estimation was taken from the heart. Glucose was determined by the method

of Hagedorn and Jensen. The liver was quickly removed, weighed and cut up into boiling 60 % potassium hydroxide, allowing 1 cc. per g. tissue. In the case of mice the livers were not weighed but directly dropped into tubes containing boiling potash and the weights determined by difference.

In young rabbits it is possible to remove quickly and cleanly the whole of the posterior thigh muscles, and such samples were invariably taken for muscle-glycogen determinations. The muscle was dropped immediately into boiling 60 % KOH. The anterior thigh and posterior leg muscles were removed for free glucose estimation, being immediately disintegrated in ice-cold absolute alcohol. In fowls a portion of the left pectoralis muscle was removed for glycogen estimation and in ferrets the procedure was to strip the leg and thigh for such samples.

Glycogen was determined by Pflüger's method, the glucose in the acid hydrolysate being estimated by the modified Bertrand method as described by Best, Hoet and Marks [1926]. Muscle-sugar was also determined according to the method used by these workers.

Results.

1. *Experiments on young rabbits.*

The experimental conditions were closely similar to those of Goldblatt's experiments on insulin alone. In each experiment a batch of healthy young rabbits from one litter was taken and kept without food for 24 hours. In each case approximately equal numbers were taken for injection with insulin and as normal controls respectively. The ages of the litters varied from 6½ to 8 weeks, and the weights of individual rabbits in the whole series from 400 to 900 g. The average weight of 11 controls was 654 g. and of 11 rabbits receiving insulin 620 g. In a certain number of his experiments Goldblatt estimated the glycogen in the gastrocnemii of each rabbit as well as that of the liver. The number of such estimates on rabbits receiving insulin alone was hardly large enough, however, to justify the attribution of much significance to the relatively small deficit of muscle-glycogen observed, as compared with that in the few controls; and Goldblatt did not, in fact, lay stress upon it. I have estimated the glycogen in muscles as well as liver in all experiments on rabbits, and, to increase the chance of obtaining representative samples, have taken muscles from the whole of the posterior thigh in each case, as described under Methods. Table I shows the results on the 22 rabbits, estimates of liver-glycogen being available in all cases, and of muscle-glycogen in all except one of the controls, in which it was lost by accident.

There is clear confirmation of the increase of liver-glycogen observed by Goldblatt as the effect of insulin; the lowest figure in the insulin group is nearly 2½ times as great as the highest in the normal group, and the average insulin value more than 7 times as great as the normal. The effect on the muscle-glycogen is not so uniform. Of the 10 normal values, 2 are so low

as to be within the general insulin range, and of the 11 insulin values 4 are high enough to be within general range of the normals. If we compare the simple averages of the two groups, that of the normal is practically twice as high as that of the insulin series. The figure in either case, however, is obviously subject to a rather wide range of error. It seemed worth while, nevertheless, to calculate for the glycogen from the liver and muscles the average total quantities per rabbit in the two groups, even though the figures could be regarded as only rough approximations.

Table I.

A. *Normal controls.*

Rabbit wt. g.	Liver wt. g.	Liver-glycogen %	Muscle-glycogen %	Muscle-sugar %	Blood-sugar %
476	13·96	0·24	0·19	0·100	0·131
480	16·08	0·25	0·18	0·099	0·163
462	14·62	0·60	0·21	0·117	0·150
464	17·28	0·20	0·30	0·127	0·141
852	19·33	0·28	(Lost)	0·115	0·152
582	15·18	0·08	0·66	0·103	0·110
800	20·30	0·12	0·42	0·127	0·106
690	20·06	0·57	0·08	0·097	0·132
904	23·97	0·42	0·12	0·103	0·122
804	24·41	0·33	0·30	0·124	0·156
676	17·55	0·22	0·27	0·112	0·138
Av. 654	18·43	0·30	0·27	0·111	0·136

B. *After insulin (0·5 unit).*

553	21·73	3·72	0·15	0·094	0·052
581	21·10	3·66	0·13	0·098	0·015
407	16·14	2·10	Trace	0·113	0·041
520	14·53	3·20	0·07	0·098	0·051
645	18·73	1·20	0·27	0·124	0·061
715	20·84	2·73	0·24	0·117	0·051
860	23·25	2·03	0·19	0·112	0·056
672	21·53	1·90	0·04	0·095	0·072
521	15·83	1·47	0·08	0·074	0·050
748	19·98	1·33	0·20	0·115	0·043
598	18·47	1·00	0·12	0·092	0·057
Av. 620	19·28	2·22	0·14	0·103	0·050

The livers were weighed in all cases, but a figure was required for estimating the weight of muscle from the total weight of a rabbit. Best, Hoet and Marks found that the muscles of a cat contributed approximately one-half of the total weight of the intact animal. Estimates were made on two young rabbits by the methods which they used.

Exp. Two young rabbits were kept fasting for 24 hours, weighed and then killed. The skin, the viscera of the abdomen and thorax and the brain were removed and weighed. The difference represented roughly the weight of muscles and bones. The stripped carcase was then boiled, and the bones were cleaned and weighed. The difference again gave the weight of the muscles. Table II shows the results.

Table II.

Rabbit wt. (g.)	809	797
Muscles and bones (g.)	504	482
Bones (g.)	106	79
Muscles (g.)	398	403
Wt. of muscles %	49	50

The muscle weight of the young rabbits, like that of the cat, can accordingly be taken to be about one-half the total body weight. Applying this factor, and the known average weight of the livers, we obtain figures for the average totals of liver- and muscle-glycogen in the two series, as shown in Table III.

Table III.

Averages	Normal	After insulin	Difference
Wt. of animal (g.)	654	620	—
,, muscles (g.)	327	310	—
,, liver (g.)	18·43	19·28	—
Liver-glycogen %	0·30	2·22	—
Muscle-glycogen %	0·27	0·14	—
Total liver-glycogen (g.)	0·055	0·428	+0·373
,, muscle-glycogen (g.)	0·882	0·434	−0·448
,, glycogen (g.)	0·937	0·862	−0·075

It will be seen that, on this calculation, the total loss of muscle-glycogen under insulin is actually slightly greater than the total gain of liver-glycogen. As already indicated, the figures for muscle-glycogen are too irregular and the method of calculation, involving a large and only approximate factor for weight of muscle, too rough to warrant the attribution of any significance to this difference. It is, however, justifiable to conclude that the gain of liver-glycogen, though much easier to detect with certainty, is not a more important item in the total change produced than the loss from the muscles.

Taken by itself, such a result would still be quite compatible with Goldblatt's suggestion as to the mechanism of insulin action. The normal level of the blood-glucose being maintained by liberation at an appropriate rate from the liver-glycogen, a stoppage of this process would lead the blood-sugar to fall, and the hypoglycaemia might in its turn prevent the muscle from replenishing the glycogen used in contractile metabolism. On the other hand, this combination of effects—the disappearance of glycogen from the muscles and its accumulation in the liver—is the direct reverse of that described by Cori and Cori as produced by insulin in the rat. Such a contrast clearly suggests the desirability of examining the effects in some other species.

2. *Experiments on chickens.*

All the chickens used were from a strain of the Plymouth Rock variety bred at the Institute's Farm Laboratories, Mill Hill, and were of a known and uniform age of about 3 months from hatching. The normal well-fed chicken, as others have recorded, has an apparent percentage of blood-glucose, as determined by reduction, about twice as great as that of the average mammal. It is stated that the true percentage of glucose is some 30 mg. lower than

this reduction-value, which is partly due to uric acid, ergothioneine and other non-carbohydrate reducing substances. I have made no attempt to differentiate, recording the total reduction-values in all cases as glucose. It should be noted, however, that if an absolute correction of the amount above indicated were applicable to the figures for "blood-sugar" after insulin, a condition of very severe hypoglycaemia would in many cases be indicated, though no pronounced symptoms, and certainly nothing suggesting convulsions, occurred as the result.

The figures in Table IV, obtained from 3 chickens not deprived of food up to the time of killing, give an idea of the range of the "blood-sugar" and liver-glycogen in such normal birds.

Table IV. *Normally fed chickens.*

"Blood-sugar" %	Liver-glycogen %
0·199	3·64
0·200	2·77
0·188	2·4

It was found, however, that fasting for 24 hours sufficed to reduce the liver-glycogen to a very low proportion, so that in most cases it was below the range of trustworthy estimate by Pflüger's method, and had to be recorded as an unmeasured "trace." Chickens so prepared should accordingly offer an experimental material closely comparable to the young rabbits used in Goldblatt's experiments and my own. Birds, as others have observed, being very resistant to insulin, it was necessary to give it in relatively enormous doses to produce definite hypoglycaemic symptoms, 80 units per bird were injected in hourly doses of 20 units each, and the birds were killed when distinct symptoms of inco-ordination had appeared after the last dose. Table V shows the results on the glycogen content of the liver and muscles of these birds.

Table V. *Chickens after 24 hours' fasting.*

Controls Percentages			Insulin Percentages		
Blood-sugar	Liver-glycogen	Muscle-glycogen	Blood-sugar	Liver-glycogen	Muscle-glycogen
0·199	Trace	0·50	0·052	Trace	0·51
0·175	,,	0·26	0·068	,,	0·32
0·200	,,	(Lost)	0·084	,,	0·61
0·188	,,	0·31	0·052	,,	0·85
0·199	0·26	0·43	0·052	,,	0·60
0·199	0·18	0·40	0·068	0·32	0·50
	Av.	0·38		Av.	0·56

It will be seen that, in complete contrast to the effect on young rabbits, there is here no sign of accumulation of glycogen in the liver as the result of the action of insulin. The glycogen of the muscles, on the other hand, shows a small, but probably significant increase under the action of insulin.

The conditions of absorption from the alimentary canal, as also of new formation of carbohydrate in the liver, might of course be different in the chicken and the rabbit, when both were subjected to fasting for 24 hours. It was, therefore, of interest to study the effects on the glycogen of the liver and of the muscles of giving known amounts of glucose by the mouth, with and without insulin, to chickens which had previously fasted for 24 hours. Several experiments of this kind were carried out, and, since the conditions in all were identical, and the results uniform in direction, the latter may conveniently be presented together, as in Table VI. It will be seen that 5 birds were kept fasting as controls, 9 were given glucose alone, in each case 3 g. as 20 cc. of a 15 % solution by tube into the crop, and another 9 the same treatment with glucose and 40 units of insulin hypodermically, 20 units being given at the same time as the glucose, and another 20 units an hour later. Two hours after the administration of the glucose the birds were killed for analysis.

Table VI.

	Fasting controls Percentages			3 g. glucose by mouth Percentages			3 g. glucose by mouth 40 units insulin hypodermically Percentages		
	Blood-glucose	Liver-glycogen	Muscle-glycogen	Blood-glucose	Liver-glycogen	Muscle-glycogen	Blood-glucose	Liver-glycogen	Muscle-glycogen
	0·184	0·18	0·73	0·226	1·95	0·38	0·251	0·08	0·81
	0·199	Trace	0·90	0·234	2·45	0·52	0·343	0·58	0·78
	0·190	0·22	0·49	0·213	2·28	0·73	0·164	0·29	1·08
	0·206	Trace	0·75	0·232	2·12	1·00	0·163	0·55	1·03
	0·206	,,	0·22	0·232	2·28	0·41	0·152	Trace	(Lost)
Av.	0·197		0·62	0·240	1·81	0·40	0·141	2·02	1·01
				0·243	1·90	0·62	0·102	Trace	0·62
				0·228	2·70	0·85	0·286	,,	0·48
				0·282	1·70	0·94	0·127	0·15	0·85
			Av.	0·237	2·02	0·65	0·192		0·83

The results, as regards the liver-glycogen are perfectly clear. Glucose alone by mouth, without extraneous insulin, causes a rapid replenishment of the glycogen store of the liver, depleted by fasting. This is accompanied by a moderate rise in the average level of the blood-sugar. When insulin is given in addition to the glucose, no definite hypoglycaemia occurs, some values being above, others below, the normal and the average being practically the same as in the controls. Nevertheless, except in one aberrant case, the insulin can be said to have prevented, or restricted to very small dimensions, the effect which the same dose of glucose by itself produced on the liver-glycogen. As usual, the figures for muscle-glycogen are more irregular, and the comparison of averages has a consequently less certain validity. They appear to indicate that, whereas glucose alone causes no perceptible change in the glycogen content of the muscles, insulin in addition to glucose causes some increase in the muscle-glycogen, whereas it prevents, as a rule, the accumulation in the liver which glucose alone causes.

Taking these results as they stand, and deferring for the moment any discussion of the processes concerned, we may note that the effects of insulin in the fowl, whether glucose is also given or not, resemble those described by Cori and Cori in the rat, and are in sharp contrast to those observed by Goldblatt and by myself in the rabbit.

3. *Experiments on young ferrets.*

These were chosen as being another type of animal, intermediate in size between the rabbit and the rat, purely carnivorous, and easily obtainable in litters of sufficient size. A few preliminary experiments showed that the liver of the ferret retains its glycogen store with great tenacity, from 2 to 4 % being present after 24 hours' fast, and that the species has a relatively high resistance to insulin, from 12 to 15 units being necessary to produce a recognisable hypoglycaemia in a young ferret. Even with such doses the only symptoms were muscular weakness and inco-ordination; no convulsions were seen.

Six young ferrets of the same litter, 7½ weeks old, were taken for an experiment. All were kept fasting for 24 hours. Three were then set aside as controls, and each of the other 3 received 12 units of insulin hypodermically. Three hours later all the treated animals were visibly flaccid and their movements inco-ordinate. All 6 were killed, and determinations made of blood-sugar, liver-glycogen and muscle-glycogen. The results are shown in Table VII.

Table VII.

	Controls Percentages			After insulin Percentages		
	Blood-sugar	Liver-glycogen	Muscle-glycogen	Blood-sugar	Liver-glycogen	Muscle-glycogen
	0·102	2·4	0·23	0·052	2·4	0·42
	0·092	3·8	0·31	0·052	1·9	0·45
	0·092	2·8	0·25	0·058	3·9	0·48
Av.	0·095	3·0	0·26	0·054	2·7	0·45

It will be seen that the insulin has produced a definite, though not extreme, hypoglycaemia. The liver-glycogen shows no significant change. The increase of muscle-glycogen, on the other hand, is consistent, and of significant dimensions.

4. *Experiments on mice.*

The effect of insulin on the distribution of glycogen between the liver and the muscles of the rat had been very thoroughly studied by Cori and Cori, and it seemed unnecessary to make further experiments on this animal. In mice, on the other hand, the early experiments of Dudley and Marrian, which first drew attention to the unexpected disappearance of glycogen under insulin action, were few in number. Lesser and his colleagues used a method which did not differentiate between liver and muscles. Further experiments on

mice seemed, therefore, desirable and, in particular, it seemed important to discover whether insulin in any dose, in any relation to food, and at any stage of its action, could be shown to promote such storage of glycogen in the liver of the mouse as it does in that of the young rabbit. Under no conditions, however, has it been possible to demonstrate such an effect, even as a preliminary phase of the action. In some cases the muscle-glycogen was estimated by removing the viscera, the head and the skin, and working up the muscles and bones of the stripped carcase together.

Exp. Ten mice weighing 16 to 20 g. were kept without food for 12 hours. Five were used as controls and each of the other five received 0·05 unit of insulin subcutaneously. The mice were kept at the laboratory temperature, so that the effect of insulin was protracted. The first of the insulin mice showed symptoms of weakness after 70 minutes and was killed, with one of the controls. Thereafter as each injected mouse showed such symptoms, and before any sign of convulsions appeared, it was killed, together with a control mouse. In each case the liver was removed, and dropped into hot potash, and the carcase similarly treated. The 5 control livers were worked up together, and similarly the 5 insulin livers. Separate estimates were made of the carcase-glycogen and of the sugar in the blood taken from each animal at killing. The last mouse was killed about 90 minutes after the insulin was given. The results are shown in Table VIII.

Table VIII.

	Controls Percentages			After insulin Percentages		
	Blood-sugar	Liver-glycogen (Av.)	Carcase-glycogen	Blood-sugar	Liver-glycogen (Av.)	Carcase-glycogen
	0·168		0·10	(Lost)		0·12
	0·131		0·22	0·041		0·08
	(Lost)	2·1	0·15	0·026	None	0·08
	0·113		0·08	0·024		0·07
	0·140		0·12	0·034		0·07
Av.	0·138		0·15	0·031		0·08

Though the minute "glycogen" precipitate from the combined 5 insulin livers was duly collected and hydrolysed, it did not yield a titratable amount of glucose. The carcase-glycogen shows a probably significant reduction, but has not approached exhaustion.

Exp. Twenty mice were kept without food for 12 hours. Ten were kept as controls and each of the other 10 received 0·02 unit of insulin. All the mice were then placed in a thermostat at 37°, at which temperature the smaller dose of insulin caused symptoms of hypoglycaemia in 15 to 25 minutes. The insulin mice were, as before, killed as they reached the stage of preconvulsive symptoms, and the controls alternately with them. The blood samples and the livers of each group were pooled for analysis, so that the figures obtained and shown in Table IX are average percentages.

Table IX.

Controls		After insulin	
Blood-sugar	Liver-glycogen	Blood-sugar	Liver-glycogen
0·139	1·11	0·034	0·003

Again the liver is practically emptied of glycogen. Several larger series of mice were then taken, which had been feeding up to the time of starting the observation or, in one series, until 2 hours before that time. In each series 5 were taken as controls, and the remainder received 0·05 unit of insulin. All were kept at room temperature, the insulin mice being killed in batches of five at intervals varying from 5 minutes up to 1 hour after the injections. The glycogen percentage was estimated on the pooled livers of each such sample of 5 mice. The results are given in Table X.

Table X. *Liver-glycogen percentages.*

No. of mice	Controls	Time after injection of insulin			
		5 mins.	10 mins.	15 mins.	1 hr.
15	1·67	—	—	0·91	Trace
15	2·54	2·34	—	—	0·01
20	2·07	2·03	1·56	—	Trace

In these well-fed mice, also, there is no suggestion of increased storage of liver-glycogen at any stage. Five minutes after the injection, with the dose only partly absorbed, the liver-glycogen is not certainly diminished, but shows no sign of increase; in 10 minutes the decrease is becoming obvious; after an hour there is practically no glycogen left.

A further similar series of measurements was made on 20 mice which had been kept without food for 16 hours. After 15 had each received 0·02 unit of insulin, they were kept in the thermostat at 37° and killed in batches up to 25 minutes from the time of injection. Table XI shows the percentages of liver-glycogen.

Table XI.

	Controls	5 mins.	10 mins.	25 mins.
Liver-glycogen %	0·30	0·13	0·15	0·001

Again, the first perceptible effect of insulin is a diminution of the liver-glycogen, exhaustion being practically complete in 25 minutes under these conditions. These experiments are typical of a number of others in which the effect of insulin was tested on mice fully fed, or fasting for different periods, and killed for examination at varying intervals after the injection, from 5 minutes onwards. On a total of 310 mice, in no case was any increase of liver-glycogen detected, but always a decrease when a significant change had occurred.

Bissinger, Lesser and Zipf made their observations on mice which, after fasting for 18 hours, were given insulin with 50 mg. glucose. Under those conditions they observed an initial increase in the glycogen of the whole mouse, occurring at an earlier period than in similar mice receiving glucose

alone. It seemed desirable to attempt to repeat this observation, and to estimate the glycogen separately in the liver and in the stripped carcase, consisting chiefly of muscle.

Exp. Eight mice were accordingly kept without food for 18 hours, after which 80 mg. of glucose were injected into each mouse, and 0·04 unit of insulin into each of four, the others being kept as controls. After 30 minutes all were killed and worked up in the usual manner, the livers in each group being pooled, and the carcases examined separately. Table XII shows the result.

Table XII.

Controls Percentages			After insulin Percentages		
Blood-sugar	Liver-glycogen (Av.)	Carcase-glycogen	Blood-sugar	Liver-glycogen (Av.)	Carcase-glycogen
0·104		0·099	0·103		0·11
0·160	0·15	0·091	0·170	0·08	0·13
0·106		0·055	0·160		0·11
—		0·051	0·101		0·11
	Av.	0·074		Av.	0·12

It will be seen that in this series the supply of glucose by injection has been adequate to prevent, in every case, a fall of the blood-sugar in the mice receiving insulin below the range observed in the controls. The muscles under these conditions show a gain of glycogen, small indeed, but with such regularity that it is probably significant. In the pooled livers, on the other hand, in spite of the maintained blood-sugar, the glycogen has fallen to a value corresponding to an average content of about one-half that in the controls, although the latter is itself already remarkably low.

Experiments on young rabbits with adrenaline.

In certain of his experiments on rabbits Goldblatt gave insulin and adrenaline. He evidently assumed that adrenaline by itself would lessen the glycogen in the liver, and that storage under the combined action, such as he observed, furnished evidence of the prepotency of insulin, keeping glycogen in the liver in spite of the stimulus of adrenaline to its discharge.

Earlier evidence had, indeed, given us clear warrant for the assumption that the action of adrenaline with regard to liver-glycogen is simply to promote its discharge as glucose, whether in the rabbit or other species. There was a body of evidence in favour of its promoting, under suitable conditions, a new formation of carbohydrate in the liver, as well as discharge of what was already stored; so that the balance of affect, whether gain or loss of glycogen, might differ according to the depleted or loaded state of the cells when the adrenaline was given.

Goldblatt's results with insulin on rabbits, however, which my own have closely confirmed, were so unlike those obtained on other species, either by

myself or by other observers, as by Cori and Cori on rats, that it seemed desirable to make a few direct observations on the effects of adrenaline on the glycogen distribution in young rabbits, under the conditions of Goldblatt's and my own experiments with insulin.

Exp. Four young rabbits of the same litter, 8½ weeks old, were kept without food for 24 hours. Two were kept as controls, and the other two each received 0·75 mg. of adrenaline, given as three hypodermic injections of 0·25 mg. each at intervals of 20 minutes. Two hours after the first injection the animals were killed, and the liver and samples of the musculature worked up as usual. Table XIII shows that the well-marked hyperglycaemia was accompanied by loss of glycogen from the liver, and also, though in smaller degree, from the muscles.

Table XIII.

Controls Percentages				After adrenaline Percentages			
Wt. of rabbit g.	Liver-glycogen	Muscle-glycogen	Blood-glucose	Wt. of rabbit g.	Liver-glycogen	Muscle-glycogen	Blood-glucose
1066	0·36	0·21	0·152	1058	0·03	0·11	0·268
1051	0·51	0·13	0·148	1021	0·02	0·06	0·320

Rabbits treated with such a relatively large dose of adrenaline are, of course, losing carbohydrate from the system through glycosuria. In Cori and Cori's experiments on rats, the doses of adrenaline which caused increase of liver-glycogen were such as produced no conspicuous hyperglycaemia. An attempt was made to produce similar conditions in young rabbits.

Exp. Two series were taken, 8 from 1 litter and 6 from another, 4 and 3 being kept as controls. The treatment of the others was exactly the same as in the former experiment, with the exception that the 4 larger rabbits of the first series received 0·15 to 0·17 mg. adrenaline each, and the 3 smaller ones of the second series received 0·1 mg. each. Table XIV shows the combined results.

Table XIV.

Controls Percentages				After adrenaline Percentages				
Wt. of rabbit g.	Liver-glycogen	Muscle-glycogen	Blood-glucose	Wt. of rabbit g.	Liver-glycogen	Muscle-glycogen	Blood-glucose	Adrenaline dose mg.
1020	0·45	—	0·135	1015	1·87	—	0·168	0·17
686	0·46	0·55	0·141	164	0·92	0·36	0·215	0·15
576	0·46	0·44	0·145	798 (1)	0·46	0·22	0·252	0·16
722	(Lost)	0·40	0·138	886	1·60	0·19	0·155	0·17
500	0·10	0·46	0·148	492	1·94	0·16	0·163	0·10
535	0·13	0·40	0·120	509 (2)	0·61	0·20	0·148	0·10
495	0·10	0·34	—	558	1·40	0·13	0·163	0·10
Av.	0·28	0·43		Av.	1·26	0·21		

It will be seen that in every case except one, which is marked (1) in the table, the liver-glycogen is definitely higher than in any of the controls. In this instance it will be seen that the blood-glucose is raised to the level of a definite hyperglycaemia, only slightly below the range of those in Table XII.

In another instance, marked (2) in the table, the increase of liver-glycogen is relatively small in relation to the highest value of all the controls, but it is still some 5 or 6 times as abundant as in the controls of its own small group. In this case the blood-sugar is hardly above the normal range, and imperfect absorption of the injected adrenaline may, perhaps, be suspected. Even when these cases are included, the average liver-glycogen content of the 7 rabbits treated with adrenaline is 4 or 5 times as great as that in 6 available controls. The increase is almost as striking as that recorded by Goldblatt, and by myself, with insulin. The muscle-glycogen in the treated rabbits is again reduced to about one-half that in the controls.

Discussion.

If we look at the results here recorded as a whole, it is obvious that they cannot easily be interpreted on Goldblatt's supposition that inhibition of the discharge of glycogen from the liver is the primary and essential effect of insulin, to which all others are secondary. They show that the effect on which he based this suggestion, namely the increase of glycogen in the liver of the young rabbit, is an unusual rather than a constant and typical effect of insulin on the normal animal. In none of the other species here under experiment have I succeeded in detecting it at all: in the mouse, in which the most systematic effort was made to discover it as a temporary phase, I failed, even when sufficient glucose was given to prevent a fall of blood-sugar.

A comparison of the results of Tables I and XIV might suggest another possibility. Results immediately similar to those in Table XIV were obtained by the Coris in young rats also, with small doses of adrenaline; but in rats and in the species other than rabbits here examined insulin has been shown to produce a shift of the glycogen balance in the other direction, the muscles gaining and the liver losing glycogen. Is it possible, then, that the glycogen changes seen in the rabbit are due not to insulin at all, but to adrenaline secondarily secreted in response to it? There are many facts which forbid the assumption of so complete a paradox. Adrenaline, given in such a dose that we can safely regard it as acting in substantial excess, causes not storage but discharge of glycogen from the liver (cf. Table XIII). In an animal without a pancreas, and therefore without insulin, glycogen disappears from the liver, to be stored again when insulin is given.

These anomalies and apparent contradictions are familiar to anyone who has followed the investigations which, during the past 8 years, have been stimulated by the discovery of insulin. With the evidence before us of the readiness with which secretion of insulin is provoked by a rise and adrenaline by a fall of the blood-sugar, we may well hesitate before too confidently attributing the effects on glycogen distribution, following the injection of either of these hormones into a normal animal of any species, to the uncomplicated action of the one injected. To obtain a safe basis, however, for even a speculative estimate of the respective parts played by the two hormones

in the production of any particular result, we must turn to the evidence provided by experiments of a kind enabling the action of each to be studied in isolation. For the effects on muscle-glycogen such evidence is relatively complete, and simple in its significance. Insulin by itself causes increase of the muscle-glycogen at the expense of circulating glucose [Best, Dale, Hoet and Marks, 1926]. Adrenaline by itself, as suggested by experiments of Cori and Cori on rats, and confirmed in experiments on the eviscerated spinal cat by Marks and myself, which are being separately published, causes discharge of glycogen from the muscle, but not as glucose. Accordingly, when injection of insulin causes hypoglycaemia accompanied by increase of muscle-glycogen, we may reasonably conclude that insulin itself is the predominant agent; when insulin hypoglycaemia, in the absence of convulsions, is accompanied by loss of muscle-glycogen, as in the young rabbit, we may reasonably suspect that secretion of adrenaline has caused glycogen to be discharged from the muscles faster than it is stored under the action of insulin.

The evidence as to the action of insulin and of adrenaline separately on the liver is not so easy to interpret. The restoration of glycogen to the diabetic liver by insulin naturally aroused the expectation that such an effect would be demonstrable on the isolated liver with artificial perfusion. With insulin alone attempts to detect this action have uniformly failed. It may properly be suggested that the maintenance of a physiological condition under artificial perfusion is peculiarly difficult in the case of the liver cells. This, however, will hardly account for the fact that a slow increase in the glycogen of a perfused liver may be retarded or converted into a decrease, but never accelerated, by addition of insulin to the blood [Bodo and Marks, 1928]. On the other hand, it is a matter of common experience that adrenaline accelerates the discharge of glycogen as glucose from a perfused liver. If the facts are valid, they seem to lead to the difficult conclusion that both insulin and adrenaline cause disappearance of glycogen from the liver, and that neither promotes its storage. There are, however, certain observations on record which, though they need critical repetition and extension, are at least suggestive.

v. Issekutz [1924, 1927] detected no direct action of insulin alone on the rate of breakdown of glycogen into sugar in the perfused livers of frogs and rabbits. Certain of his experiments, however, suggest an antagonism of insulin to the mobilising effect of adrenaline on liver-glycogen, and some even appear to show that, when insulin alone has failed to affect the output of glucose, adrenaline in addition may retard it. Cori and Cori have interpreted the storage of glycogen in the liver of the normal rat, following the injection of small doses of adrenaline, with concurrent loss from the muscles, as due to passage of lactic acid from the muscles, and its resynthesis to glycogen in the liver; but it is at least open to doubt whether this gain by the liver would occur in the absence of the pancreas. We are entitled, then, to consider the possibility that, when either insulin or adrenaline acts alone, or in great

excess, it causes a diminution of liver-glycogen—either by increasing the rate of discharge in relation to that of new formation, as we can reasonably assume in the case of adrenaline, or by depressing the rate of new formation in relation to that of discharge, which is compatible with what is known of the action of insulin—but that some kind of balanced action of both is needed for the normal storage of glycogen in the liver. Such a view would certainly meet the case of the diabetic animal, where the liver, emptied of its glycogen, in spite of extravagant gluconeogenesis, under the unbalanced action of adrenaline[1], regains its normal store and rate of production when insulin is given in the dosage needed to restore the balance. In the normal young rabbit, again, we have found reason, from the effect of an insulin injection on the muscle-glycogen, to suspect that the action of insulin itself is complicated, to a greater extent than in other animals, by that of adrenaline secreted in response to it; and it is in this species alone, of those here examined, that storage of liver-glycogen has been found to follow injection of insulin. It may be doubted whether the whole of an effect following the injection of insulin into any normal animal can safely be attributed to insulin alone; but the storage of glycogen in the rabbit's liver seems to be more clearly under suspicion of secondary complication than most. The possibility that yet other hormones than adrenaline may play a part in effects following an injection of insulin must be kept in view, but there are no experimental data for its discussion.

Summary.

1. The accumulation of glycogen in the liver of the young, fasting rabbit as the result of injecting insulin, described by Goldblatt, is confirmed.

2. Under such conditions the musculature loses at least as much glycogen as the liver gains.

3. Similar effects have not been observed in other species. Insulin causes no deposition of glycogen in the liver of the fasting chicken, but prevents its deposition there as the result of giving glucose, while causing its deposition in the muscles. In the ferret it causes formation of glycogen in the muscles and has little effect on that in the liver. In the mouse it causes rapid discharge of glycogen from the liver; when glucose is also given, glycogen is deposited in the muscles, but still disappears from the liver.

4. Injections of adrenaline too small to cause glycosuria produce, in the young, fasting rabbit, a change in glycogen distribution closely similar to that following injections of insulin.

5. It is argued that the effects of insulin on the normal animal are complicated by effects of adrenaline secreted in response to its action, especially in the rabbit.

It is a pleasure to express my gratitude to Dr Dale for his constant interest and criticism during the investigation.

[1] The term "adrenaline" is used for convenience, to include also possible similar actions by sympathetic nerves to the liver.

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LXXXVIII. THE COMPARATIVE RATES OF ABSORPTION OF SUGARS FROM THE HUMAN INTESTINE.

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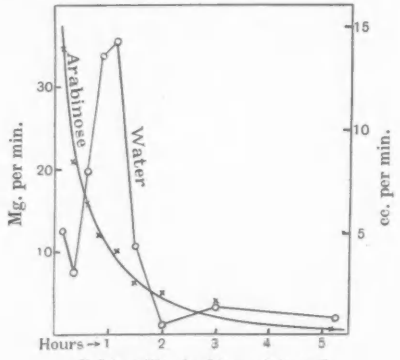
Hoppe-Seyler [1881] and Heidenhein [1894] first drew attention to the fact that simple physical laws could not explain the absorptive phenomena in the intestine. Höber [1899] compared the rates of absorption of several sugars and salts and found that dogs absorbed galactose slightly faster than glucose. Hédon [1900] compared the rates of absorption of glucose, fructose, galactose and arabinose from a loop of rabbit's gut. He found that from a 25 % solution arabinose was absorbed most quickly and then in order galactose, glucose and fructose. From isotonic solutions glucose was absorbed most rapidly. Working with "Vella" fistulae on a series of four dogs Nagano [1902] found that the monoses showed characteristic relative rates of absorption. Galactose was most rapidly absorbed and then in order glucose, fructose, mannose, xylose and arabinose. Solutions over 10 % were not used. Hewitt [1924] compared the rates of absorption of dilute solutions of glucose, fructose and galactose from loops of gut. In rabbits no very conclusive results were obtained, but in cats glucose was absorbed much more rapidly than fructose, while galactose occupied an intermediate position. All were absorbed at equal rates after killing the intestinal mucosa by hot liquids or sodium fluoride. Cori [1925], using standard rats, administered by stomach-tube a strong solution of the sugar to be tested and subsequently killed the animals after varying intervals, and the sugar still left in the whole of the alimentary tract was estimated. This method was an improvement on the older ones in that intact animals could be employed. Large numbers of rats moreover could be used and the results averaged. Cori placed the sugars in the same order as Nagano and found their relative rates (glucose = 100) to be: galactose 110, glucose 100, fructose 43, mannose 19, xylose 15, and arabinose 9. Macleod [1929] has recently criticised one or two points in Cori's technique, and Magee and Macleod [1929] have determined the rates of diffusion of sugars through isolated oxygenated loops of rabbits' intestine. No more definite conclusions were drawn than that hexoses were absorbed more rapidly than pentoses.

[1] Working with a part-time grant from the Medical Research Council.

Clearly none of the methods so far described is applicable to man, but we have devised a method by which the rates of absorption of rhamnose, arabinose and xylose have been compared. These three sugars are all excreted readily by the kidney and are only comparatively slowly destroyed in the tissues. We have therefore compared the rate and amount of each sugar excreted (*a*) when injected intravenously, (*b*) when taken by the mouth.

The sugars used were naturally occurring ones and were obtained from The British Drug Houses, Ltd. The xylose and arabinose each contained 0·2 % ash and 0·2 % moisture and were at least 98 % pure. The rhamnose contained 0·3 % ash and 15·8 % moisture which was allowed for in making the weighings. Dried, it melted at 121–125°. The estimations were all carried out by McCance's methods [1926, 1929]. Three normal adult men have been the subjects, H. age 23, wt. 64·5 kg.; H.L.S. age 25, wt. 57·2 kg.; R.A.M. age 30, wt. 66 kg. The procedure on each has been identical.



Subject H. Arabinose injected.

Fig. 1. Each point is plotted in the centre of the period during which that particular specimen of urine was secreted. It indicates in mg. per min. and cc. per min. the average rate of excretion of arabinose or water over that period of time.

Experimental.

The effect of diuresis upon excretion.

Our experiments early established the fact that a moderate diuresis made little or no difference to the rate or amount of the sugar excreted. Fig. 1 illustrates this by comparing the rates of excretion of water and arabinose by subject H. The pentose was injected. Assuming the pentoses to be non-threshold bodies and that they are filtered off in the glomeruli we take this to mean that a diuresis of this degree does not involve the opening up of fresh glomerulo-tubular units to the circulating blood, but merely re-absorption of less water in the tubules [Rehberg, 1926]. Sometimes with small frequent samples of urine we have observed an increased output of sugar to accompany a small increase in urinary volume. This we have only observed when the

rate of excretion was falling, and we regard it as being technical in origin. It is not always easy to empty the bladder completely at short intervals and a small amount of urine left behind would be voided with the next specimen and explain the experimental finding.

Injection experiments.

5 g. of the sugar to be tested were dissolved in 15 cc. of distilled water and sterilised at 100°. The whole of this solution was then injected slowly into the antecubital vein, the time taken for the actual injection being 4 mins. Zero time for the experiment was taken to be 2 mins. after the injection had begun. No tourniquet was applied. The bladder was emptied just before the

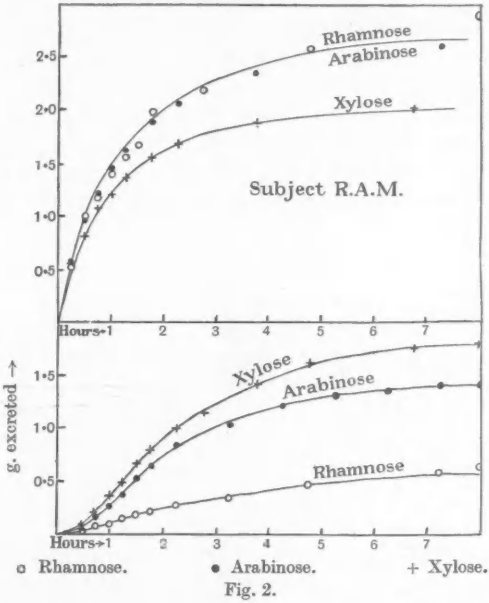


Fig. 2.

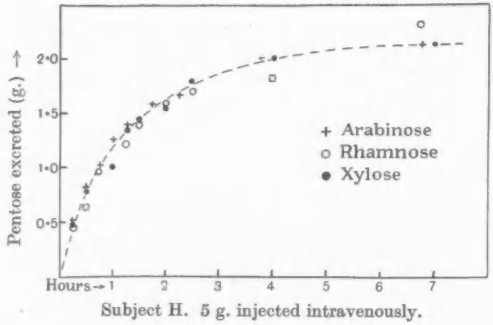
injection and again every 15 mins. for 1¼ hours: later at extending intervals up to 7 or 8 hours when the experiment terminated. These experiments always began at about 10 a.m. and the subject had had a light breakfast. Small amounts of water were drunk at intervals. A moderate lunch of cheese and bread was taken about 1.30 p.m. at which water only was drunk. Fig. 2 (upper half) shows the detailed results from one subject (R.A.M.). The excretion curves of rhamnose and arabinose are almost identical. The rate is slower and the amount of xylose excreted less than that of the other two, but the shape of the curve is the same. In the case of subject H. the excretion curves of all three sugars were superimposable and are reproduced in Fig. 3. Differences in the rate and amounts of excretion (as in the case of xylose above)

are presumably due to differences in the rate of utilisation by the tissues (see later).

There is no evidence that pentoses are stored as such in the animal body, and therefore these injected sugars can only be destroyed or excreted. The amount destroyed (or used) in any experiment can therefore be found by deducting the total amount excreted from the amount injected, *i.e.* 5 g.

Ingestion experiments.

Each subject took 5 g. of sugar by mouth and the excretion was again followed as before. After an overnight fast the subject emptied his bladder and drank 100 cc. water in which the sugar was dissolved. Urine was collected at the end of the first half hour and then for 1½ hours at 15 minute intervals. Thereafter collections were made at longer intervals up to 7 or 8 hours. Small amounts of water were taken from time to time. Fig. 2 (lower half) shows the actual results obtained from one subject (R.A.M.). These curves should be compared with those obtained from the same subject after the sugars had been injected. The initial delay in excretion shown by all three sugars is not due to the temporary delay of the solution in the stomach as will be apparent later. It should be noted that, whereas xylose is here excreted in greater amount than either of the others, less was excreted on injection.



Subject H. 5 g. injected intravenously.

Fig. 3.

Rhamnose is very poorly excreted when taken by the mouth, though readily after injection, which confirms injection experiments of Voit [1897]. On the other hand the relative amounts of these three sugars excreted by our subjects do not correspond to those of Von Jaksch [1899].

From these curves we obtain for each sugar the total amount excreted after ingestion. From the injection experiments we have obtained the characteristic ratio $\frac{\text{sugar used}}{\text{sugar excreted}}$ for each sugar. By applying it we obtain the amount used in these ingestion experiments. The amount used + the amount excreted = the amount absorbed. In this way we can calculate the amount of each sugar which has reached the tissues in the time of the experiment.

Results.

By applying this method we have obtained the following results which may be put into tabular form.

Table I.

Injection experiments.

	Total amount excreted after injection of 5 g.			Total amount used			Ratio $\frac{\text{sugar used}}{\text{sugar excreted}}$		
Subject	Rhamnose	Arabinose	Xylose	Rhamnose	Arabinose	Xylose	Rhamnose	Arabinose	Xylose
R.A.M.	2·8	2·65	2·1	2·2	2·35	2·9	0·79	0·9	1·38
H.L.S.	2·3	2·7	2·3	2·7	2·3	2·7	1·17	0·79	1·17
H.	2·3	2·2	2·2	2·7	2·8	2·8	1·17	1·27	1·27
Average	2·5	2·5	2·2	2·5	2·5	2·8	1·0	1·0	1·27

Ingestion experiments.

	Total excreted			Total used			Amount absorbed		
	Rhamnose	Arabinose	Xylose	Rhamnose	Arabinose	Xylose	Rhamnose	Arabinose	Xylose
R.A.M.	0·62	1·6	1·8	0·49	1·4	2·5	1·1	3·0	4·3
H.L.S.	0·40	1·2	1·65	0·47	0·95	1·9	0·87	2·15	3·55
H.	0·82	1·5	2·2	0·96	1·9	2·8	1·78	3·4	5·0
Average	0·60	1·4	1·9	0·6	1·4	2·4	1·2	2·8	4·3

The consistency of these results is very satisfactory. It will be noted that subject H. absorbed more of all the sugars than the others and H.L.S. less. R.A.M. absorbed intermediate amounts of each sugar. If the rate of absorption of rhamnose is taken to be 1 the comparative rates become rhamnose: arabinose : xylose, 1 : 2·33 : 3·6. We may note in passing that the very small amounts of rhamnose absorbed explain the high coefficients of utilisation found by many of the pioneers on this subject [Lindeman and May, 1896].

Experiments with rats.

We decided to repeat some of Cori's experiments on xylose and arabinose and extend them to rhamnose. The rats used were obtained from Vitamins, Ltd. and were all adult males of an inbred strain weighing between 230 and 300 g. each. Four rats were used for each sugar in two lots of two each and the procedure was as follows. 2 g. sugar were dissolved in a small quantity of distilled water and the volume was made up to 5 cc. $3\frac{1}{2}$ ins. of a No. 3 flexible rubber catheter was attached to the nozzle of a 2 cc. "Record" syringe and the whole was filled with sugar solution up to the 2 cc. mark. A rat was then lightly anaesthetised with ether, the catheter passed quickly into the stomach and 2 cc. discharged from the syringe. In this way each rat received 0·8 g. After 3 hours the rats were killed by coal gas and the whole alimentary canal was at once dissected out and placed in a beaker, covered with a little water and boiled. The intestines were then cut up into small fragments

with scissors and extracted repeatedly with boiling water. The washings were made up to 200 cc. and the sugar was estimated. The amount so found deducted from 0·8 g. gives the amount absorbed.

Note. Both Cori and Macleod have considered the possibility that the intestinal flora may utilise the sugars sufficiently fast to invalidate this method of obtaining the amount of sugar absorbed. Cori's controls show that the bacterial action is negligible.

Table II.

Sugar	Amount absorbed (g.)				Average (g.)	Comparative rates rhamnose = 1	
						Rats	Man
Rhamnose	0·04	0·20	0·07	0·15	0·12	1	1
Arabinose	0·23	0·30	0·23	0·25	0·25	2·1	2·33
Xylose	0·45	0·47	0·44	0·40	0·44	3·67	3·6

The relative rates in man have been added to the table for comparison and it is unnecessary to emphasise the agreement. The relative rates of absorption of arabinose and xylose found by Cori, when reduced to our scale become 2·1 : 3·5, results almost identical with our own.

There can be little doubt that the epithelium of the human intestine reacts to sugars in the same way as that of the rat. The relative rates of absorption of rhamnose, arabinose and xylose have been shown experimentally to be the same in both. We may assume with considerable confidence that the comparative rates of absorption of glucose, galactose and fructose found in rats will also hold in man. It is probable also that the same relative rates hold for the dog [Nagano, 1902], but our knowledge of this very fundamental relationship between sugars on the one hand and intestinal epithelium on the other is regrettably incomplete. The limited experiments on rabbits have not been consistent. We do no even know, therefore, whether this peculiar relationship holds throughout the mammalia, much less at what stage in evolutionary history it appeared.

The kinetics of sugar absorption.

1. So far we have dealt only with the total amounts of sugar excreted or absorbed. Fig. 4 now shows the varying rates of excretion of all three sugars following their ingestion by H.L.S. It is at once evident that the peak of excretion occurs about 1 hour after they have been swallowed, whatever the sugar taken and whatever percentage of that sugar is ultimately to be excreted. This we interpret to mean that these sugars are all absorbed best at the same level in the intestine and that this level is high up near the pylorus.

2. *The actual absorption curves.* It is evident that the curves of excretion shown in Figs. 2 and 4 in no way resemble the actual curves of absorption. Sugar continues to be excreted long after it has ceased to be absorbed because the amounts temporarily stored in the fluids of the body are only slowly utilised or excreted. The fact that excretion is not yet quite finished

8 or 9 hours after 5 g. have been injected is proof of this. The curves of absorption can however be constructed from the following considerations. The amount absorbed up to any given moment is equal to the sum of:

(1) the amount excreted up to that moment;
(2) the amount used up to that moment;
(3) the amount still in the "tissues" at that moment.

The first is obtained by direct estimation. The second is obtained as follows. Assuming that the rate of excretion and the rate of destruction (or utilisation) are both proportional to the amount in the body at any given time, then the ratio $\frac{\text{sugar used}}{\text{sugar excreted}}$ is a constant whatever the time. We have already made use of this ratio to calculate the total amount of each sugar used. It can be employed in the same way to calculate the amount used at any given time. The third, *i.e.* the amount in the tissues at any moment, is proportional to the rate of excretion at that moment and can be calculated from it.

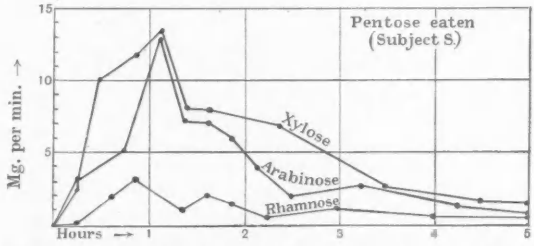
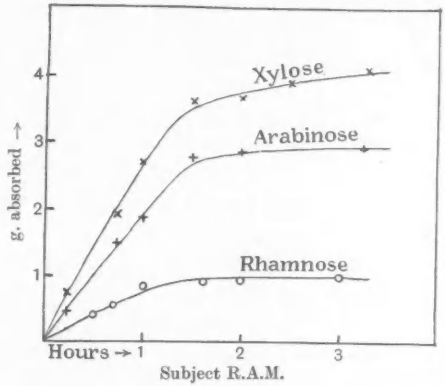


Fig. 4. Each point is plotted in the centre of the period during which that particular specimen of urine was secreted. It indicates in mg. per min. the average rate of excretion of sugar over that period of time.



Subject R.A.M.
Fig. 5.

The actual curves of absorption of subject R.A.M. are shown in Fig. 5, and should be contrasted with the curves of excretion of the same subject (Fig. 2, lower half). The exact determination of one of the points is given for

the sake of clarity. The characteristic ratio $\frac{\text{sugar used}}{\text{sugar excreted}}$ for each sugar has been determined from the smoothed experimental curves and is for xylose 1·38 and for arabinose and rhamnose 0·82.

To calculate the amount of arabinose absorbed 1 hour after ingestion, proceed as follows:

(1) The amount excreted up to 1 hr. (by direct estimation) = 0·25 g.
(2) The amount used up to 1 hr. = 0·25 × 0·82 = 0·19 g.
(3) The amount in the tissues.

The amount excreted between ¾ hr. and 1 hr. after ingestion = 0·1 g. (by direct estimation); the amount similarly excreted between 1 and 1¼ hrs. = 0·09 g. Assuming the rate of excretion to be constant for this half hour, the rate of excretion per hour at 1 hr. after ingestion = 0·38 g. From the smoothed curve of excretion after injection this rate of excretion is found 2·05 hrs. from the start and at this time 1·95 g. have been excreted and therefore 1·95 × 0·82 = 1·6 have been used.

Therefore 5 − (1·95 + 1·6) = the amount in the tissues corresponding to a rate of excretion of 0·38 g. = 1·45.

Therefore the amount in the tissues 1 hr. after ingestion = 1·45 g.

Total amount absorbed = 1·905 g.

Great accuracy cannot be expected from these curves. In the first place they are based upon the assumption that the sugars distribute themselves equally throughout the body as soon as they are absorbed. This cannot be strictly true, but they certainly leave the blood stream and diffuse into the organs with great rapidity, for after 5 g. have been injected intravenously the curve of excretion is smooth and regular from the start. There is no excess of excretion in the first 10 mins. No serious error therefore, is involved in this assumption. Secondly, the determination of rates in this manner is not strictly accurate, particularly over parts of the curve where the rate is changing fast, *e.g.* between ½ and ¾ hour after ingestion. At the same time the samples of urine were collected over short periods (15 mins.), and the curves are extremely regular, so that there can be no doubt of the general truth of the results.

It is striking that the absorption of all these sugars should proceed rapidly (at a linear rate) for about 1½ hours and then almost or entirely cease when large amounts of arabinose and rhamnose presumably still remain in the gut. It is clear that absorption must commence as soon as the sugar is ingested and—as has already been pointed out—the upper part of the small intestine is the site of absorption. The lag in excretion is due to the time taken for the concentration in the tissues to rise.

Cori found that the rate of absorption in rats proceeded at a linear rate for 3 hours if enough sugar was given [Cori, Cori and Goltz, 1929; Pierce, Osgood and Polanski, 1929]. This Cori appears to consider a function of the epithelium. Cori's published results are very convincing and there can be little doubt that they are correct. Our own calculated absorption curves also show this tendency to a linear rate of absorption, at any rate at first, and it is to be recalled that we gave relatively very small amounts of sugar.

We would attribute the steady linear rate of absorption in Cori's cases (and in our own) not to a function of the epithelium, but to a slow steady

emptying of the stomach. With rhamnose in our subjects absorption ceased while four-fifths of what was taken still remained in the intestine, and a steady linear rate of absorption could only have been obtained by a slow steady supply of fresh sugar to the absorbing areas in the upper part of the small intestine. The sudden cessation of absorption at 1½ hours in our experiments we take to mean that after this time the stomach was empty and no fresh sugar was being passed down the intestine. It is significant that there was always sugar left in the stomach of Cori's rats.

Note. By making certain assumptions it is possible to calculate the rate of filtration through the glomeruli directly from the curves of sugar excretion following injection. Thus in Fig. 2 the rate of excretion after 2 hrs. = 0·38 g. per hr. Assuming this rate to be constant for 15 mins. this would lead to the excretion of $\frac{0{\cdot}38}{4} = 0{\cdot}095$ g.

1·95 g. have been excreted at this time. Therefore, $1{\cdot}95 \times 0{\cdot}82$ have been used = 1·6 and so $5 - (1{\cdot}95 + 1{\cdot}6)$ g. remain in the body = 1·45. Assuming the fluid content of the body to be 60 % of the weight, this subject contains about 40 litres, and assuming further the pentose not excreted to be uniformly distributed, the concentration of pentose in body fluids is 3·6 mg. per 100 cc., so that 2660 cc. must have been filtered off to give the 0·095 g. excreted, *i.e.* 178 cc. per minute.

Similar calculations from other curves give amounts of filtration varying between 100 and 170. The example given in detail is an exceptionally high one.

It is perhaps not quite legitimate to assume that the pentose sugars distribute themselves equally throughout the cells of the body, for Kozawa [1914] found that human red blood-corpuscles were impermeable to rhamnose although permeable to xylose and arabinose. Cori and Goltz [1925], however, have found that pentoses penetrate the tissues as readily and freely as hexoses, and the similarity of our excretion curves for the three sugars also indicates that this is true of the three pentoses. Accurate work on this subject would necessarily require estimations of pentose in the plasma.

These figures for glomerular filtration agree with those obtained by Rehberg [1926].

Summary.

1. Rhamnose, arabinose and xylose are excreted equally readily when injected intravenously in man. Their curves of excretion are of identical shape and may all be superimposable.

2. The relative rates of absorption of rhamnose, arabinose, and xylose are the same both in rats and men. If the rate of absorption of rhamnose = 1, those of arabinose and xylose are 2·33 and 3·6 respectively.

3. These sugars are all absorbed at the same level in the intestine and this level is high up in the small intestine. Lower down little or no absorption takes place.

4. In normal man absorption of these sugars proceeds rapidly and at a linear rate for 1½ hours and then almost ceases, even when large excess still remains in the intestine.

5. We may assume with reasonable confidence that the relative rates of absorption of glucose, galactose and fructose found in rats also hold in man, *i.e.* galactose is absorbed slightly faster than glucose, and the latter twice as fast as fructose.

We wish to express our thanks to Mr J. B. S. Haldane for some very valuable criticism.

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LXXXIX. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

IV. A FURTHER STUDY OF THE SERIES OF RYVOSH.

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The second paper of this series [Ponder, 1927] was concerned with an investigation, by quantitative methods outlined in the first paper of the series [Ponder, 1926], of the statement that the order of resistance of the red cells of various animals to saponin is the reverse of the order of resistance to hypotonic saline. The result of the study was to confirm the conclusion of Ryvosh [1907] that the following series holds for hypotonic saline, the most resistant species occurring first: man, guinea-pig, rat, rabbit, dog, pig, cat, ox, goat, sheep, and that the order of resistance to saponin is nearly the reverse. The same results have been more recently obtained by one of us [Yeager, 1928] and the facts thus seem to have been placed beyond question.

The explanation of the facts, however, is a very much more difficult matter. To make this clear, we shall tabulate the various suggestions which have been advanced.

(i) The resistance of red cells to saponin is stated to be determined by the quantity of cholesterol contained in them, and the lytic action of saponin to be due to its power of combining with this substance ([Yagi, 1911] and others).

(ii) Port [1922] has suggested that the phosphoric acid content of the cell is the essential factor in determining the resistance, a statement which is accepted by Orahovats [1926] and borne out by the recent analyses of Kay [1928].

(iii) Brinkman's theory regarding the resistance of cells to lysis by hypotonic saline implies that the resistance becomes greater as the cholesterol/lecithin ratio becomes greater [Brinkman, 1920], from which we may deduce that the resistance to saponin haemolysis becomes greater as this ratio decreases. This statement is incompatible with (i). Further, the conclusion of Meyer [1908] that red cells are the more resistant to saponin the higher the cholesterol/lecithin ratio, directly contradicts Brinkman's explanation.

(iv) In the second paper of this series we have suggested that saponin reacts with the cell proteins, and that the high negative correlation between

phosphoric acid content and resistance to saponin is due to there being a correlation of + 0·79 between resistance and protein content (Abderhalden's figures [1895], not particularly reliable in this connection), and one of − 0·75 between phosphoric acid content and protein content.

(v) The resistance of red cells to digitonin haemolysis is stated to be parallel to the cholesterol content of the cell [Fabre and Simonnet, 1926]. This statement corresponds to (i), made for saponin.

(vi) Kofler and Lázár [1927] have shown that the order of resistance of various cells to saponin is by no means the same as the order to digitonin and to other lysins. A new series, in fact, arises with each lysin. This is certainly true so far as the bacterial haemolysins are concerned [Ponder and McLachlan, 1927], and one of us [Yeager, 1928] has shown that the resistance series for sodium taurocholate is quite different from that for saponin.

The difficulty associated with any of these suggestions as to the cell component with which the lysins combine is due to the obvious fact that if (vi) is correct, no one of the above suggestions can be used to explain the action of more than one haemolysin. For example, if (vi) is true, either (i) or (v), or both, must be wrong, for it is impossible for the action of two lysins to be determined by the quantity of the same component and yet for the two lysins to be uncorrelated with each other with respect to their activities. Similarly, if we explain the action of saponin in terms of (ii), and if (vi) be correct, the same explanation cannot be used to account for the action of the related lysin digitonin, or of any other lysin which gives rise to a resistance series other than that obtained with saponin.

The extension of the Ryvosh series.

The following table shows the resistances of the washed red cells of various mammals to saponin and to hypotonic saline. The measurements of resistance were carried out exactly as described in the second paper of this series, and might properly be added to Table I of that paper, as the suspensions contained the cells from an equal volume of blood.

Table I.

Animal	Saponin R	Saline %	Animal	Saponin R	Saline %
Marmoset ...	0·57	0·40	Macaque ...	1·55	0·22
Baboon ...	0·59	0·18	Chimpanzee	1·70	0·28
Lemur ...	0·64	0·42	Armadillo	2·10	0·31
Squirrel ...	0·90	0·27	Racoon ...	3·0	0·32
Green monkey	0·90	0·43	Kangaroo	3·5	0·32
Man	1·00	0·32	Skunk ...	3·7	0·39
Opossum ...	1·10	0·35	Wallaby ...	4·2	0·38
Spider monkey	1·24	0·28	Black buck	6·0	0·46
Sapajou „	1·42	0·38	Buffalo ...	7·0	0·42
Elephant ...	1·5	0·28	Elk ...	8·0	0·42

As can be seen from a mere inspection of the figures, there is no obvious relation between the resistance to saponin and the resistance to hypotonic saline. The coefficient of correlation, in fact, is − 0·50, and that of rank corre-

lation even lower, about $-0{\cdot}3$[1]. It thus appears that the conclusions of Ryvosh cannot be extended to the red cells of the mammalia in general, although they are correct for the animals included in the original series. We must consider how this can come about.

The essential point is that the ordinary theory of correlation applies only where large numbers are concerned and where random sampling is approached. Suppose, for the sake of argument, that the red cells of the various mammals are tabulated with respect to their resistance to saponin and to hypotonic saline, and that there is zero correlation between these two attributes, we shall, nevertheless, by a judicious choice of animals, be able to pick a small series which shows a high positive (or negative) correlation between the resistances referred to. For instance, from the 29 animals included in the above table and Table I of the previous paper, we can select the following series for resistance to saponin and to hypotonic saline respectively (least resistance first).

Hypotonic saline: sheep, goat, ox, cat, pig, dog, rabbit, rat, guinea-pig, man.

Saponin: rabbit, rat, man, guinea-pig, dog, pig, cat, goat, ox, sheep.

The rank correlation shown here is $-0{\cdot}88 \pm 0{\cdot}071$. Or we may select the following series (least resistance first).

Hypotonic saline: green monkey, lemur, marmoset, sapajou, racoon, man, armadillo, elephant, macaque.

Saponin: marmoset, lemur, green monkey, man, sapajou, elephant, macaque, armadillo, racoon.

The rank correlation shown here is $+0{\cdot}70 \pm 0{\cdot}17$, a result almost exactly opposite to that of the first series.

These two coefficients are different in sign, and both cannot possibly indicate the true state of affairs in the population they purport to describe; so it must, therefore, be possible for either to arise by chance from the particular population under consideration. The question is whether we can assess the chance. Where small numbers are concerned, there is no way of estimating the chance of a high correlation coefficient, positive or negative, arising from a large population showing zero correlation, for the usual tests, in which we compute the number of times the standard error is exceeded, fail altogether in cases such as this, where the true standard error of the coefficients is unknown. The standard errors of $\pm 0{\cdot}07$ and $\pm 0{\cdot}17$, attached to the correlation coefficients above, are, in fact, quite misleading, for they are calculated on the erroneous assumption that the numbers are large; if they were correct, the chances of the two coefficients arising by chance from a population showing

[1] Calculation of coefficients of correlation, real or rank, from such a small number of cases as those dealt with in this paper is, of course, unsound statistical practice, and the coefficients have very little meaning. They are useful, nevertheless, for expressing a relation between two attributes, when the relation is not evident by simple inspection. As describing such a relation, even with small numbers, such coefficients are quite satisfactory: the danger in their use arises from the fact that we cannot assume them to be efficient statistics for a population including any cases additional to those from which they were obtained.

zero correlation would be one in millions. The very fact that, out of a series of 29 animals, two such coefficients can be selected, shows that no significance can be attached to either as describing the true state of affairs in all the animals.

The series of Ryvosh accordingly appears to constitute a special case, involving the animals which, for economic reasons, are usually used in laboratories. This does not mean, of course, that there is actually zero correlation between resistance to saponin and resistance to hypotonic saline, for, were sufficiently extensive series examined, a significant negative correlation might still be found; on the existing evidence, however, we cannot say that such a correlation exists, and we may go even further and say that it is probable that it does not.

The cell component determining resistance to saponin.

The fact that the cells of different animals vary some twenty times in resistance to saponin has suggested to many investigators that it may be possible to show that the varying quantity of some one substance accounts for the variation in resistance. The substances which have been suggested are (i) cholesterol, (ii) phosphates, and (iii) lecithin together with cholesterol, *i.e.* the cholesterol/lecithin ratio.

(i) The hypothesis that saponin produces lysis by combining with cholesterol rests upon several pieces of evidence: (*a*) that there is a high correlation between the cholesterol determinations of Abderhalden and resistance to saponin; (*b*) that digitonin, a substance allied to saponin, precipitates cholesterol quantitatively; (*c*) that Ransom [1901] and others have stated that the inhibitory action of serum on saponin haemolysis is due to the contained cholesterol uniting with the saponin and rendering it inert. We may place against these observations the following. (*a*) The analyses of Abderhalden give figures for total cholesterol, including esters which are not precipitated by saponin or digitonin, and are therefore rather misleading in this connection. (*b*) Kofler and Schrutka [1925] have measured the affinity of eleven kinds of saponin for cholesterol, and have shown that the combining power of saponin for this substance is by no means parallel to the lytic power. (*c*) Several observers have shown that the inhibitory action of serum on saponin haemolysis is due principally to its contained proteins [Ponder, 1923; Kennedy, 1925].

(ii) The hypothesis that saponin produces lysis by reacting with the phosphates (or with phosphate-containing substances) rests upon the fact that the coefficient of rank correlation between phosphoric acid content and resistance to saponin is very high ($-0{\cdot}83$, with Abderhalden's figures, or $-0{\cdot}92$ with Kay's figures). The appearance of the negative sign, however, completely eliminates the possibility of the lysin reacting with the source of the phosphoric acid, or of using the phosphoric acid content as a guide to the mode of action of the lysin, for the following reason. The whole of recent work indicates that haemolysis by saponin can be treated quantitatively on the assumption that,

to produce lysis, a certain quantity of saponin, x, must be used up in combining with the cell membrane. The method of measuring resistance is such that the resistance of the cell increases as x increases, and hence as the substance attacked by the lysin increases. If the quantity of any substance in the cell membrane decreases as x increases, and if it is contended that the quantity of the substance determines lysis, the correlation coefficient has a negative sign; the meaning of the sign is that, whatever substance be attacked by the lysin, it is not this one.

(iii) The conflicting statements regarding the cholesterol/lecithin ratio have already been remarked upon.

The experiments hitherto done in this connection have generally failed to conform to one or all of three important conditions. (i) The usual methods for determining the resistance of cells to saponin really determine only the resistance of the most resistant cells, whereas chemical analyses determine the amount of substances contained in cells of average composition. Ideally, we should compare the content of the average cell with the resistance of the average cell population. (ii) The suspensions of the cells of various animals used in determining resistance must all contain the same number of cells, for, if the number is not the same, comparison of the results becomes almost impossible[1]. The quantity of a given substance obtainable from this number of cells can, however, easily be calculated from analyses on any known number of cells. (iii) It is important that resistance and chemical composition be determined for the cells of the same animal, and that analyses of the cells of one individual of the species shall not be compared with the resistance of the cells of another individual of the species, for the variation is great. It is not necessary, however, to employ large numbers of animals of the same species, for the resistance of the cells of one individual depends (by hypothesis) upon the amount of a given substance in its own cells, and certainly not, on any conceivable hypothesis, on the amount of the substance in the cells of other individuals.

With these points in mind, we have carried out a series of experiments in the following way. A set of dilutions of quillia saponin in 0·85 % sodium chloride, from 1 in 1000 to 1 in 560,000, was made in the usual way and stored in a refrigerator, being removed only when actually to be used. Blood was obtained from the animal (sheep, ox, horse, man, dog, pig, rat, rabbit and guinea-pig), the cells washed three times in 0·85 % NaCl and then made up to the original volume of the blood from which they were obtained. This suspension was used for the estimation of the following substances: (*a*) cholesterol (method of Myers and Wardell), (*b*) inorganic phosphates (Benedict and Theis), (*c*) total acid-soluble phosphates (Fiske and Subbarow) and (*d*) lecithin-phosphorus (Bloor, Benedict and Theis). The results were then reduced to quantities present per $0{\cdot}5 \times 10^6$ cells/mm.[3]

[1] The reason for this is that increasing the number of cells does not result in an equal increase in the quantity of saponin apparently used up. The increase in the latter figure is proportional to that in the former, but the proportionality constant is difficult to determine.

A small portion of the suspension was used for a cell count and a part of the suspension was afterwards diluted so as to give a suspension containing $0{\cdot}5 \times 10^6$ cells/mm.^{3}, which is of a convenient strength for the determination of resistance to saponin. The resistances were determined by finding the quantity of lysin necessary to produce 50 % haemolysis after 7 hours at 25°. All the experiments performed were completed within 2 weeks, thereby ensuring that the activity of the lysin remained unchanged.

The results may be described briefly as follows. (*a*) The coefficient of rank correlation between resistance to saponin and quantity of cholesterol is small and negative (− 0·6), and there is no evidence that the resistance increases with the quantity of contained free cholesterol. The results, in fact, lead to the same conclusions as those of Kofler and Schrutka. (*b*) The figures for total phosphates agree excellently with those of Kay. The correlation between the quantity of phosphates and resistance to saponin is again high but negative (− 0·75). Although the quantity of contained total phosphates may be a satisfactory guide to the resistance of the cell, the negative sign precludes the possibility of the haemolytic reaction being dependent, except indirectly, on the quantity of phosphates. (*c*) The coefficient correlating inorganic phosphates and resistance is also high but negative (− 0·80). (*d*) The cholesterol/lecithin ratio is not significantly correlated with resistance to hypotonic saline and therefore cannot be correlated positively or negatively in a significant manner with resistance to saponin (+ 0·45 to saline, − 0·30 to saponin). The coefficients of rank correlation above given are, of course, of no absolute value, but will serve as an incomplete description of the relation between the two attributes concerned.

The haemolytic activity of related lysins.

The hypotheses hitherto discussed in this paper, *i.e.* those of Yagi, and Fabre and Simonnet regarding cholesterol, of Port regarding phosphates, and of Meyer and Brinkman regarding the cholesterol/lecithin ratio, have in common the fundamental assumption that the total quantity of some substance contained in the red cell membrane determines the resistance to lysis—that is, the minimal quantity of lysin required for complete lysis. If we accept this idea, and also suppose that chemically related haemolysins act upon the same component, it then follows that the cells of a series of animals arranged in the order of their resistance to one of the lysins are also of necessity arranged in order of their resistance to all the other lysins.

The only data on the subject of closely related lysins are those of Kofler and Lázár, and these agree with the result of observations on chemically unrelated lysins in showing that quite different resistance series arise when different lysins are used. The animals in Kofler and Lázár's experiments were sheep, ox, horse, man, dog, pig, white rat, rabbit, and guinea-pig, and Table II shows the order of resistance to different lysins, the figure 1 indicating the greatest resistance.

Table II.

	Saponin	Digitonin	Smilacin	Helleborein	Senegin	Gypsophila saponin
Sheep ...	1	9	8	3	2	1
Ox ...	2	8	9	4	5	3
Horse ...	3	4	7	7	9	2
Man ...	4	3	2	5	1	5
Dog ...	5	2	5	1	7	4
Pig ...	6	7	3	2	8	7
Rat ...	7	1	1	6	4	9
Rabbit ...	8	5	4	8	6	6
Guinea-pig	9	6	6	9	3	8
Coefficient of rank correlation		− 0·34	− 0·57	+ 0·52	+ 0·7	+ 0·88

As can be seen from inspection of the figures, and as is indicated by the coefficients of rank correlation between the resistance to saponin and to each of the other lysins, there is no uniform order of resistance.

In view of the importance of this conclusion, we have repeated the experiments using quillia saponin, digitonin and smilacin, and have determined the concentration required to produce 50 % haemolysis of a suspension containing $0{\cdot}5 \times 10^6$ cells/mm.^{3}. The orders of increasing resistance are shown in Table III, bracketed animals having as nearly the same resistance as can be determined experimentally; to Table III is also added the resistance series obtained with sodium taurocholate [Yeager, 1928] and with the lysins produced by *B. tetani* and by *B. histolyticus* [Ponder and McLachlan, 1927].

Table III.

Saponin	Digitonin	Smilacin	Sodium taurocholate	Lysin of *B. tetani*	Lysin of *B. histolyticus*
Rabbit	Sheep	Sheep	Guinea-pig	Rabbit	Sheep
Pig	Rabbit	Horse	Sheep	Ox	Ox
Rat	Ox	Ox	Dog	Horse	Rabbit
Man	Man	Pig	Rabbit	Man	Man
Guinea-pig	Pig	Dog	Rat	Sheep	Horse
	Cat	Rabbit	Ox		
Dog	Rat	Rat	Man		
Horse	Dog	Cat	Cat		
Cat	Guinea-pig	Guinea-pig	Pig		
Sheep		Man			
Ox	Horse				

The results in the first three columns confirm the conclusion of Kofler and Lázár, and show, as do the results in the last three columns, that the resistance series is different with each lysin. It should be observed that in the case of the first three lysins the order of resistance of the cells of the different animals is not identical with that found by those investigators; this is no doubt due to the fact that two samples of quillia saponin, smilacin and even of digitonin are often not entirely comparable[1], owing to their uncertain composition, and the same remark might well apply to two preparations of the bacterial lysins. The failure to obtain the same resistance series as Kofler and Lázár, however, leads to the conclusion that different orders are obtained not only with different lysins, but sometimes even with different samples of what is thought to be the same lysin.

[1] Different specimens of digitonin, although stated to be chemically pure, have ,for example, different effects on the heart.

Since there is conclusive evidence that the cells of animals arranged in order of their resistance to one lysin are not also arranged in order of their resistance to other lysins, it follows that the haemolytic action of different lysins cannot be determined simply by the varying quantities of a single cell component, *e.g.* we cannot imagine the action of quillia saponin, digitonin and helleborein to be determined by the quantity of free cholesterol, cholesterol ester, phosphate, or any other substance contained in the cell membrane. It should be particularly observed that the nature of the cell component whose quantity is imagined to determine the resistance is of no importance, for the same argument would apply whatever its nature and whatever the affinity of the haemolysin may be for the substance, since varying affinities would not change the order of resistance.

If this conception is rejected, as it has to be, there remains the possibility that the resistance to a given haemolysin depends on the total quantity of a cell component, but that for each lysin a different component must be postulated. This explanation, more particularly, demands that there shall be as many components as there are resistance series, and it is possible to imagine such a number only by postulating that such compounds as "protein" are really made up of numerous different proteins, each one of which reacts specifically with each one of the many lysins. Speculations in this direction are fruitless at the present time.

The subject is much more profitably approached in the following way. In studies of the kinetics of haemolysis by simple haemolysins, the only postulate which has been found necessary is that the lysin acts on a cell component S and transforms a portion of it S_1, sufficient to make permeability to haemoglobin complete[1]. The resistance is measured in terms of this quantity S_1, or rather in terms of a quantity which is proportional to it, the quantity x of lysin used up in the process. This quantity x is directly obtainable from the figure for the smallest concentration (or the greatest dilution) required to produce a given amount of haemolysis, say 50 %, and, like the quantity S_1, has no necessary or even probable relation to the total quantity of S, except that S_1 cannot be greater than S.

It follows at once that there is no necessary or even probable relation between resistance and the total quantity of any cell component which may be mentioned. The suggestions of Port, Yagi, Meyer, Fabre and Simonnet, Brinkman and others really imply that S_1 always is equal to S, or is a simple function of S. The idea that such a relation exists is not only unsupported by evidence, but, as we have seen, leads to the conclusion that every haemolytic reaction is unique, in the sense that every lysin which presents a new resistance series must be imagined to act on a different substance.

When a lysin acts on S and transforms S_1 of it, the new compound is

[1] In the discussion which follows, it should be noted that the hypothesis advanced differs from all others in assuming that all lysins attack only the one component S in any given type of cell; the nature of S, however, differs from one type of cell to another.

proportional in quantity to S_1, but its nature depends on (*a*) the nature of S_1 (which may be imagined to be a protein, different in different animals), and on (*b*) the nature of the lysin concerned in the transformation. The new substance S_2 which has resulted from the action of the lysin on S_1 is now a component of the membrane, just as S_1 was previously, and the permeability of the membrane depends upon (*a*) the quantity of S_2 and (*b*) its nature.

To make clear the results which follow from those considerations, let us imagine four cases. (*a*) When destruction of semi-permeability is produced, the compound formed when saponin acts on human cells may be 20 micrograms in quantity, and its nature may be denoted by H-saponin, (*b*) the compound formed with ox cells may be 80 micrograms in quantity, and in nature O-saponin, (*c*) the compound formed with human cells and digitonin may be 80 micrograms in quantity and in nature H-digitonin and (*d*) the compound formed with ox cells and digitonin may be 20 micrograms in quantity, and in nature O-digitonin. Then the same result, *i.e.* destruction of semi-permeability is produced by 20 micrograms of H-saponin in human cells, but by 80 micrograms of H-digitonin in the same cells. This difference is easily understood when we remember that the compounds H-saponin and H-digitonin are different, and may, therefore, affect permeability to an unequal extent. But further, the same result is produced in ox cells by 80 micrograms of O-saponin and by 20 micrograms of O-digitonin. This difference is equally easily understood, for the two compounds are different. The figures for ox cells, furthermore, are not necessarily the same or in the same order as those for human cells, for not only is H not the same as O, but the human cell in which the compounds H-saponin and H-digitonin are formed is different in many other respects from the ox-cells, and the effect on permeability cannot, therefore, be expected to be the same in the two cases, even for the same lysin. *A fortiori*, it cannot be expected to be the same for two different lysins.

In short, the condition demanded by the existence of different resistance series is that the resistance of any cell to any lysin, whether denoted as a figure in micrograms, or as a relative resistance R, shall be a function both of the type of cell and of the nature of the lysin, *i.e.* $R = f$ (cell, lysin). The hypothesis proposed above meets this condition, for it states that $R = f(S_2)$ and $S_2 = f$ (cell, lysin). On the other hand, a hypothesis which assumes that lysis is determined by the total quantity of some one cell component, *e.g.* cholesterol, if extended to cover more than one lysin, fails to meet the required condition because it assumes that $R = f(S_1)$, the substance acted on, rather than $f(S)_2$, the substance formed as a result of the action, and, although S_1 is a function of the type of cell, it is not a function of the type of lysin. Furthermore, the hypothesis proposed above has the advantage that the kinetics of the haemolytic reaction postulated would be essentially similar for all lysins, as is in fact the case.

Since the lytic effect is supposed to depend, by this hypothesis, upon the formation of a compound whose nature and effect vary with (*a*) the type of cell

protein and (*b*) the type of lysin, it is interesting to find (by calculation from Table III of Kofler and Lázár's paper) that the variation of resistance resulting from a variation in the lysin used is 3–4 times as great as that resulting from a variation in the type of cell. This is a result quite in accordance with expectation.

Summary.

1. The conclusion of Ryvosh that resistance to hypotonic saline varies inversely with resistance to saponin in the red cells of mammals, although substantiated for the animals included in the original series, does not appear to hold when the cells of other mammals are studied, and it is doubtful whether this inverse relation is in any way a general rule.

2. Simultaneous chemical analyses and determinations of resistance to various lysins do not reveal any one substance whose total quantity can be said to determine the resistance of the cell.

3. When the cells of various animals are arranged in order of their resistance to various lysins, a different order is found to be necessary for each lysin. This finding confirms the results of Kofler and Lázár, and, although inexplicable on the assumption that the resistance of the cell is determined by the quantity of some one substance in all cells, is completely in accordance with the theory upon which studies of the kinetics of the haemolytic reaction are based.

We wish to acknowledge our indebtedness to the New York Zoological Society, and especially to Dr Charles Noback of the New York Zoological Gardens, for making it possible for us to obtain specimens of the blood of the various rare mammals mentioned in this paper.

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XC. THE DETERMINATION OF THE QUANTITY OF FREE WATER IN ERYTHROCYTES.

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THE erythrocyte can be considered as a two-phase system, free water and dissolved salts occupying the continuous phase, while water bound with haemoglobin and lipoids constitutes the dispersed phase. The total water content of the cell has been shown by drying methods to be 70 % of the entire volume of the cell, but it is of some importance, in studying the partition relations existing between corpuscles and plasma, to determine accurately the relative proportions of free and bound water to be found in the cell. "Free water" may be defined as that proportion of the cell water that is osmotically transferable and "bound water" as that proportion that is not osmotically transferable under the same experimental conditions. It will be seen that free water is then approximately equivalent to the "dispersion medium" of previous investigators, and bound water is a part of the "dispersed phase" (consisting of water bound with haemoglobin and lipoids). Two opposing conclusions have been reached by the method of determining changes in the volume of corpuscles in saline solutions of varying osmotic pressures after prolonged centrifuging. Gough [1924] finds that the free water occupies 30–35 % of the cell volume, while Ege [1927] finds a value of 60 % for the free water. These results have been examined in the present investigation.

Gough suspends washed sheep cells in saline solutions varying in strength from 0·6 % to 4 %, spins them in a water centrifuge (*ca.* 2500 r.p.m.) for 3 hours, and then measures the height of the deposit of sedimented cells in the haematocrite tubes. Plotting the strength of solution against the cell volume reached after centrifuging, Gough finds: (*a*) that the curve for increasing volume of free water abstracted with increased concentration of NaCl assumes an asymptotic form, indicating that the maximum amount of free water can be abstracted by solutions of 3 % NaCl, and (*b*) that one must exclude a volume of two-thirds of the bulk of the corpuscle as not taking any part in the osmotic equilibrium. He thus obtains a value for the free water of 30–35 %, with the concentrations of NaCl he employs.

Ege, using the same method, concludes that the free water occupies 60 % of the cell volume, and believes that the source of error in Gough's determination is incomplete sedimentation of the corpuscles when suspended in hyper-

tonic solutions. If the column of cells has not settled completely, owing to the presence of fluid in the interstices of the cells, the value for the diminished cell volume in hypertonic solutions does not represent the "true" volume, but a slightly higher value. This introduces a considerable error, according to Ege, in the amount of free water obtained from the cell[1]. Ege points out that centrifuging must be continued until the column of corpuscles is perfectly sedimented, *i.e.* until there is no interstitial fluid. The corpuscles have then attained their "true" volume as manifested by the column of blood taking on the translucent "appearance of laked blood" throughout its entire extent (Koeppe's criterion [1905]). If complete sedimentation takes place, as evidenced by the appearance of Koeppe's criterion, the value for the free water is found to be 60 % of the cell volume.

In the following experiments the question of complete sedimentation was considered and attention fixed on determining the maximum quantity of water that can be expressed from the cells (*i.e.* whether Gough's curve actually reached an asymptote in the concentration of NaCl used by him).

Exp. 1. Previous experimenters have used sheep cells freed from serum by washing three times in 0·85 % NaCl. These are then mixed thoroughly with equal volumes of NaCl solutions of varying concentrations and transferred to haematocrite tubes 10 cm. long. Similar preparations were made in this experiment, using NaCl solutions of from 3 to 20 %, and these were compared with preparations of whole unwashed blood (retaining the serum) mixed with equal quantities of the same NaCl solutions. The haematocrite tubes were spun in a high speed centrifuge (14,000 r.p.m.) for 10 minutes–1 hour. Constant volume was reached after about 12 minutes of spinning, but Koeppe's criterion was never observed even when the spinning was continued for 2 hours. The control in all cases was either washed cells (or whole blood) mixed with an equal volume of plasma. The determination is made by measuring the height of the column of sedimented cells in plasma and expressing this as percentage volume of the entire length of the column of liquid in the haematocrite tube. This is compared with the height of the sedimented column in the various saline solutions used, and the difference between readings in plasma and hypertonic saline, expressed as a percentage of the reading in plasma, gives the volume of free water transferred from the cell.

The average value for five experiments with washed cells gave 45 % free water, while the whole blood gave a value of 32 % for the free water. Similar discrepancies were noted for ox and rabbit cells, the cells washed in "isotonic" saline always giving higher values for the volume of free water. This would seem to be due to a factor hitherto neglected by previous investigators—that 0·85 % NaCl is not "isotonic" for most mammalian cells, and that many so-called "isotonic" solutions change the volume of the corpuscles by permitting

[1] Gough's estimation of the height of column of sedimented cells is much less sensitive to errors than Ege believes, and the difference between the results of the two investigators cannot be explained in this way.

an entrance of water[1]. In the following experiments, it was decided, as a consequence, to use only unwashed corpuscles, retaining the serum.

Exp. 2. The effect of time of spinning on the volume of cells was studied in an endeavour to determine the significance of the appearance of Koeppe's criterion.

Percentage volume of cells after spinning in haematocrite.

Time of spinning	5 mins.	10 mins.	12 mins.	14 mins.	16 mins.	20 mins.	30 mins.	1 hr.	2 hrs.
				Cells in 3 % NaCl.					
Ox	9·6	9·2	9·2	9·2	9·2	9·2	9·2	9·1	9·1
Sheep	15·4	15·1	15·0	15·0	15·0	15·0	14·9	14·9	14·8
Rabbit	12·3	11·8	11·8	11·8	11·8	11·8	11·8	11·6	11·6
Human	14·3	13·8	13·8	13·8	13·8	13·8	13·8	13·2	13·2
				Cells in 8 % NaCl.					
Ox	8·5	8·2	8·2	8·2	8·2	8·2	—	—	—
Sheep	14·3	13·9	13·9	13·9	13·9	—	—	—	—
Rabbit	12·3	11·7	11·7	11·7	11·6	11·6	—	—	—
Human	15·5	14·2	14·2	—	—	—	—	—	—

Constant volume was reached in some 10–12 minutes and prolonged centrifuging gave no appreciable decrease in cell volume. In no case did Koeppe's criterion appear[2], even with prolonged centrifuging with these hypertonic solutions. It would seem reasonable to expect its appearance after prolonged centrifuging at such high speed, according to Ege's results, but it was never observed, except under circumstances which will be explained later. It is important to observe the blank spaces in the table. These denote that cells in 8 % NaCl haemolysed after prolonged spinning, thus obscuring the readings, since the disintegrated cells were spread throughout the haematocrite tube and left no clear column of sedimented cells to be used as a basis for estimating cell volume.

Exp. 3. To determine the maximum quantity of water that can be expressed osmotically from the corpuscles, the cells were placed in concentrated saline solutions and spun until constant volume was reached. In many cases haemolysis took place and the cell volume could not be accurately measured on that account.

Tonicity	3 % NaCl	6 %	8 %	12 %	20 %
	Volume of free water osmotically transferred.				
Sheep	32·1	32·8	33·0	33·0	33·0
Rabbit	30·4	31·2	31·3	31·3	31·3
Human	31·3	33·1	33·2	33·2	33·2

According to theory, the amount of water leaving the cell varies with the concentration of the surrounding hypertonic fluid. Since we get no significant increase in the amount of water leaving the cell (*i.e.* since we have apparently reached the asymptote on Gough's curve relating cell volume to strength of

[1] Unpublished data of Ponder and Saslow demonstrate these points quantitatively.

[2] It has been shown by experiments of Ponder and Saslow (now in press) that Koeppe's criterion is not coincident with the volume as determined by more accurate means such as the colorimetric method.

NaCl solution used) even with such strong solutions, we may conclude that the maximum quantity of free water has been transferred from the cell. In addition, it can easily be calculated that if the interior of the cell is assumed to consist of 100 unit volumes of a 1 % solution and if the surrounding fluid is a 4 % solution, sixty unit volumes of water would have to pass from the cell to the surrounding medium for osmotic equilibrium to be reached. If, then, we obtain less than this quantity even with more strongly hypertonic solutions, the reason is not that we cannot obtain any more water from the cell, but rather that there is no more water free to leave the cell. Solutions of KCl, sucrose and glucose were also used as suspension media but were not as successful in extracting the free water from the cells as were solutions of NaCl in similar concentrations. The experiments on sheep, ox, rabbit and human corpuscles accordingly confirmed Gough's conclusion that the free water occupies 30–35 % of the cell volume, provided haemolysis did not occur.

The appearance of haemolysis in suspensions in contact with strongly hypertonic solutions or subjected to prolonged spinning in the haematocrite complicated the experiments, but suggested the possible source of Ege's high value for the free water. When haemolysis occurs, the column of sedimented cells is decreased in height by the haemolysed corpuscles streaming up along the sides of the haematocrite tube. If this continues for any length of time, the entire tube becomes filled with the contents of the haemolysed cells and at the bottom of the tube is seen a slightly denser sediment of cells which appears somewhat translucent. The translucency, however, is not due to the close packing indicating Koeppe's criterion (which Ege describes as the "appearance of haemolysed cells"), but is due to an actual haemolysis taking place. Furthermore, the height of sedimented cells when haemolysed cannot represent the true cell volume as the cell contents have been liberated throughout the entire length of the haematocrite tube, and the cell volume is therefore very much less than the true volume. Such haemolysis with apparently smaller cell volumes and, consequently, larger amounts of free water, would seem to be the source of Ege's high value for the free water to be extracted from the cell[1].

Summary.

Determination of the maximum change of volume of blood corpuscles in NaCl solutions of varying strengths gives a value of 30–35 % of the cell volume occupied by the free or osmotically transferable water contained in the corpuscle and 30–35 % for the value of the bound water, confirming Gough's figures.

[1] Ege cites experiments by the method of determining depression of the freezing point by equal volumes of water and blood corpuscle press-juice in support of his high value for the free water. This method is not a reliable means of determining the relative proportions of free and bound water as it assumes two questionable points: (i) that the bound water is inactive in producing freezing-point lowering, and (ii) that the dissolved sucrose cannot be added to the bound water as well as to the free water.

Washing cells in "isotonic" solutions, prolonged centrifuging, the occurrence of haemolysis and the use of Koeppe's criterion as indication of true cell volume are to be noted as possible sources of error in Ege's determination of 60 % free water in the cell.

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XCI. THE USE OF THE BARCROFT APPARATUS FOR THE MEASUREMENT OF TISSUE RESPIRATION.

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(*Received May 1st, 1930.*)

The Barcroft apparatus was originally designed to measure the total amount of gas evolved or absorbed by a given process. Recently however it has been widely used for the study of tissue respiration and similar processes, in which not so much the total amount of gas absorbed as the *rate* of the process is to be determined. In order that the results should be significant in such cases, it is essential to ensure that the process is not being limited by purely physical factors depending upon the apparatus itself. Two such factors are likely to introduce errors, namely, inability of the oxygen to diffuse sufficiently rapidly from the gas into the liquid phase, and insufficient rate of absorption of the carbon dioxide, produced in the respiration, by the alkali present for that purpose. In spite of the considerable amount of work which has been carried out by means of the Barcroft apparatus on tissue systems, no data on the limitations of the apparatus from this point of view have yet been published. This question is dealt with in the present paper.

Details of apparatus.

The flasks of the apparatus were of the form illustrated in Fig. 1, this being the type used in this laboratory during the past seven years. The volume of the flask is 35 cc. and 3 cc. of liquid are used in all cases. The layer of liquid is about 1·5 mm. in thickness.

A considerable improvement in the reliability of the apparatus is effected by dispensing with the usual T taps, which were very liable either to leak or to become blocked, and substituting ordinary taps in the position shown in Fig. 1.

The liquid used in the manometers was paraffin coloured with Sudan III, which proved entirely satisfactory. Clove oil is to be avoided, as it is too viscous for use when rapid oxygen uptakes are being measured. The density

of the paraffin used should of course be accurately determined before calibrating the apparatus, so as to avoid the necessity of recalibration in the event of accidental loss of the liquid. The area of cross-section of the bore of the manometer tubes was about 0·5 mm.[2]

The usual type of shaking mechanism was employed. This was normally adjusted to give a rate of shaking of 120 complete oscillations per minute, with a distance of travel of 2·5 cm., these conditions being those adopted in previous work. The shaking was not interrupted when readings were taken.

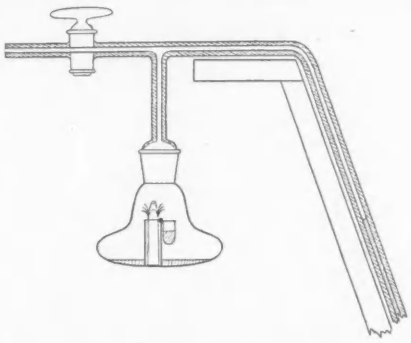

Fig. 1. Side view of apparatus. The drawing shows only one-half of the apparatus.

Absorption of carbon dioxide.

In using the Barcroft apparatus to measure the rate of oxygen uptake by tissue preparations, it is of course necessary that the effect of the CO_2 produced by the tissue shall be eliminated. For this purpose it has been customary to introduce about 0·3 cc. of a strong solution of NaOH or KOH into a small open tube sealed to the bottom of the flask, in order to absorb the CO_2. If any CO_2 accumulates in the gas phase, owing to incomplete absorption, the apparent oxygen uptake will be less than the true by a corresponding amount. If no alkali is added the movement corresponds to the difference between the oxygen absorbed and the CO_2 evolved, and this is made use of in determining the respiratory quotient (as described by Dixon and Elliott [1929]). In the course of the work just referred to we found that with the customary technique the rate of absorption of CO_2 was quite inadequate for experiments on rapidly respiring tissues. We therefore examined the efficiency of the absorption under various conditions.

For this purpose a Barcroft respirometer was set up containing in the flasks 1 mg. of sodium carbonate in the usual amount of liquid. In the right hand flask a small tube (as used by Keilin [1929]) containing 0·4 cc. of a strong solution of oxalic acid was suspended from the absorption tube by a small platinum hook as shown in Fig. 1. 40 % NaOH or KOH solution was placed in the absorption tube in the usual way. The apparatus was then

shaken in the thermostat for 5 minutes, the taps closed, and the zero reading taken. After shaking for a further period to make certain that the reading did not change, the apparatus was gently tapped so that the tube containing the acid was upset into the carbonate solution and the CO_2 (about 200 mm.^{3}) thus liberated. The CO_2 was rapidly evolved, and readings were then taken for some time after the evolution had ceased in order to follow the rate of absorption by the alkali. Results obtained for NaOH at 14° and for KOH at 40° are shown in Fig. 2, curves A and B.

It is evident from these curves that the rate of absorption of CO_2 is quite insufficient, when the customary technique is used.

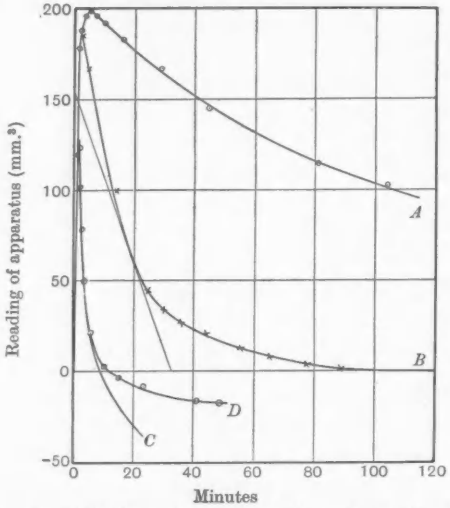

Fig. 2. Rate of absorption of CO_2 by various methods. Curve A; the absorbing agent was 40 % NaOH placed in the absorption tube; temperature 14°. (The curve for NaOH at 40° was slightly lower than this.) Curve B; 40 % KOH in the absorption tube; temperature 40°. Curves C and D; rolls of filter-paper were used in the right-hand flask only at 40°: Curve C; the paper was moistened with 40 % KOH; Curve D; 7 % KOH was used.

The tissue may be assumed to produce CO_2 at a constant rate. The rate of absorption on the other hand will depend upon the concentration of CO_2 in the gas phase. CO_2 will therefore accumulate in the gas phase until the rate of absorption becomes equal to the rate of production, and when this steady state is reached the movement of the manometer gives a true measure of the rate of oxygen uptake. Thus the error becomes smaller as the experiment progresses. It is possible to calculate the time required for attainment of the steady state and the magnitude of the error produced as follows.

Let x = the volume in mm.^{3} of CO_2 in the gas phase of the flask at any instant.

Let R = rate of evolution of CO_2 by the tissue in mm.^{3} per minute. R may be assumed to be constant.

The rate of absorption of CO_2 will be proportional to the amount of CO_2 in the gas phase. This will be the case no matter whether the rate is determined by the diffusion of the gas along the absorption tube or directly by the number of molecules striking the absorbing surface. Therefore we can write

$$\text{Rate of absorption} = cx \text{ mm.}^3 \text{ per minute,}$$

c being a constant depending on the form of the apparatus, temperature, etc.

The rate at which CO_2 accumulates is equal to the difference between the rate of evolution and the rate of absorption. Therefore

$$\frac{dx}{dt} = R - cx.$$

On integrating, and determining the constant of integration by putting $x = 0$ when $t = 0$, we get

$$x = \frac{R}{c}(1 - e^{-ct}) \qquad \text{.....(1).}$$

At $t = \infty$, $x_\infty = \frac{R}{c}$.

We may obtain the time required for x to reach 99 % of its equilibrium value by putting $x = \frac{99}{100} x_\infty$.

Then
$$\frac{99}{100}\frac{R}{c} = \frac{R}{c}(1 - e^{-ct})$$

whence
$$e^{-ct} = 0{\cdot}01$$

$$-t_{99\,\%} = \frac{-2 \times 2{\cdot}3}{c}$$

$$t_{99\,\%} = \frac{4{\cdot}6}{c} \text{ minutes.}$$

We note that the time required for x to reach 99 % of its final value is independent of R, the rate of evolution of CO_2.

By drawing a tangent to curve B, Fig. 2, at any given point we obtain the value of cx, the rate of CO_2 absorption. *E.g.* when $x = 75$ mm.^{3} the rate of absorption $cx = \frac{153 \text{ mm.}^3}{34 \text{ mins.}}$, whence

$$c = \frac{153}{34 \times 75} = \frac{1}{16{\cdot}5}.$$

Therefore $t_{99\,\%} = 4{\cdot}6 \times 16{\cdot}5 = 76$ minutes.

Suppose CO_2 is evolved at the moderate rate of 300 mm.^{3} per hour. Then $R = 5$ mm.^{3} per minute, and $x_\infty = \frac{R}{c} = 5 \times 16{\cdot}5 = 82{\cdot}5$ mm.^{3}

Using equation (1) we can plot x against t as in Fig. 3. We see that using this method of absorption, approximate equilibrium between CO_2 evolution and absorption would not be reached for 76 minutes, at which time 82 mm.^{3} of CO_2 would be present in the gas phase of the flask. Should the taps of the apparatus be closed after allowing say 10 minutes for the initial temperature equilibration, the curve shows that about 40 mm.^{3} of CO_2 are already in the flask, and therefore during the next 60 minutes a further 40 mm.^{3} of CO_2 will accumulate in the gas phase, so that the apparent oxygen uptake will be decreased by this amount.

Thus this method for CO_2 absorption is practically useless, and the following method, suggested to us by Dr Keilin, has been adopted.

A rectangle of filter-paper about 4 cm. by 2·5 cm. is rolled up fairly tightly into a cylinder, and tied with silk. The length of this roll should be about $\frac{1}{4}$ inch greater than that of the absorption tube in the flask. One end of the cylinder is then slit down by two cuts at right angles, and the ends spread out. This is then placed in the absorption tube as shown in Fig. 1, after lightly greasing the upper rim of the tube to prevent the alkali from creeping down the outside. The alkali solution is then run on to the paper.

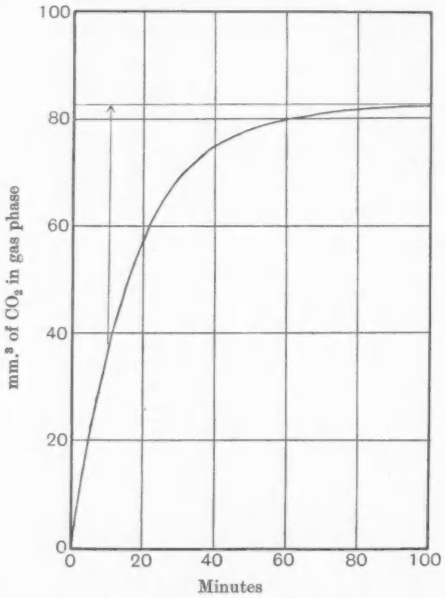

Fig. 3. The curve shows the accumulation of CO_2 in the gas phase with absorption conditions corresponding to curve B of Fig. 2, and with a CO_2 production of 300 mm.³ per hour. The length of the arrow represents the magnitude of the error produced when the taps are closed after 10 minutes.

The absorption of CO_2 is now very rapid, as is shown by curve C, Fig. 2. Using 40 % KOH at 40° it is evident however that a new factor has entered, for the amount of gas absorbed is greater than the amount of CO_2 present, and the pressure in the flask continues to fall after passing its original level. This was traced to the fact that under the action of the strong alkali the paper undergoes spontaneous oxidation with uptake of oxygen. With a weaker solution of alkali however the absorption of CO_2 is equally rapid, while the absorption of oxygen becomes nearly negligible. This is shown in curve D, Fig. 2, which was obtained using 7 % KOH at 40°. The inaccuracy due to

the oxidation of the paper can be entirely obviated by having the alkaline paper in both flasks of the apparatus. In curves A and B, Fig. 4, 6 % KOH on paper was used at 14°, the paper being in the right hand flask only in curve A and in both flasks in curve B.

By calculation similar to that given above it is found from curve B, Fig. 4, that using this method of absorption only 11 minutes is required to reach approximate equilibrium, at which time 12 mm.^{3} of CO_2 are present in the flask if the rate of production of CO_2 is 300 mm.^{3} per hour as before. If the taps are closed after 5 minutes there will be 10 mm.^{3} already present, and the amount of CO_2 will increase by only 2 mm.^{3} which is entirely negligible.

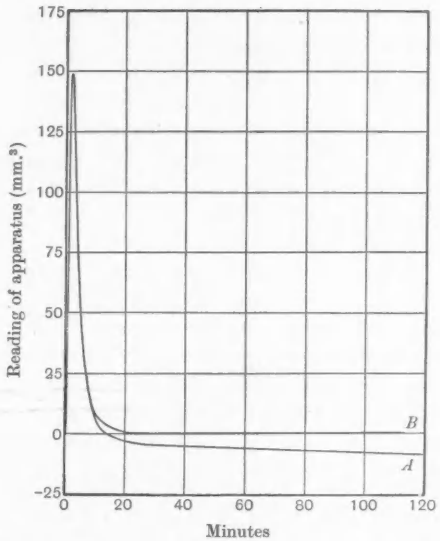

Fig. 4. This experiment was similar to that of Fig. 2. Curve A; 6 % KOH on filter-paper was placed in the right-hand flask only; Curve B; it was placed in both flasks of the apparatus. Temperature 14°.

Taking the high rate of CO_2 evolution of 1000 mm.^{3} per hour, the same calculation shows that the error when the taps are closed after 5 minutes would only be 5 mm.^{3}

This method is therefore entirely satisfactory.

Diffusion of oxygen as a limiting factor.

In order to measure the true respiration rate of cells immersed in a liquid it is necessary to ensure that the rate at which oxygen can diffuse into the liquid is rapid in comparison with its utilisation. (This applies also to the measurement of oxygen absorption by other systems.)

The importance of this was early appreciated when adapting the Barcroft apparatus for measuring rates of oxygen uptake, and Dixon and Tunnicliffe [1923] pointed out the importance of using flasks of a form exposing a large surface of liquid to the gas and giving efficient mixing of the liquid, and also of making sure in carrying out experiments that the rate of uptake is independent of the rate of shaking of the apparatus, this being an indication that the uptake is not being limited by diffusion effects. In spite of the considerable amount of work which has since been carried out with the apparatus, no quantitative data have yet been published on the rates of uptake which the apparatus is capable of measuring without errors due to diffusion effects.

The essential condition which must be fulfilled if the experiment is to be significant is that the concentration of dissolved oxygen must not fall so low that its rate of utilisation by the respiring cells (or other system undergoing oxidation) falls appreciably below that which would be obtained if the pressure of oxygen in the liquid were identical with that in the gas.

In order to make this clear let us consider what takes place when the amount of respiring cells, *e.g.* yeast, present in the 3 cc. of liquid in the flask of the apparatus is gradually increased. With very small amounts of yeast the rate of oxygen uptake will be small, and hence the pressure of oxygen dissolved in the liquid will be only slightly below that in the gas phase; the observed rate of uptake will be proportional to the amount of yeast present, and will be independent of the rate at which the apparatus is shaken (*i.e.* of the degree of mixing of the liquid). As the amount of yeast added is increased the rate of uptake is proportionately increased and the tension of dissolved oxygen will fall lower; but so long as it does not fall below the point at which the respiration rate of the yeast depends upon the oxygen pressure the rate of uptake is still a true measure of the respiration, and remains proportional to the amount of yeast and independent of the rate of shaking. A point is ultimately reached however at which the oxygen pressure in the liquid falls so low that the actual respiration rate of the yeast is cut down, when further additions of yeast have much less effect, and since the rate of uptake is now to some extent determined by the rate at which the oxygen can get to the yeast, it begins to be dependent upon the rate of shaking. Finally with large amounts of yeast the rate of uptake reaches a limiting value which is practically independent of the amount of yeast, and is entirely dependent upon the rate of shaking.

Owing to the complicated mixing conditions a quantitative mathematical treatment is hardly practicable, but we shall give here a number of experiments illustrating the effects met with and the capabilities of the apparatus.

The actual results obtained with yeast are illustrated in Fig. 5, which shows the relation between the observed velocity of oxygen uptake and the amount of yeast taken at two rates of shaking. (In all these experiments large "KOH-papers" were used, capable of absorbing the CO_2 produced by large amounts of yeast.) It will be seen that up to the point X on the curves

the apparatus measures the true respiration rate of the yeast, since the velocity of uptake remains proportional to the amount of yeast taken up to this point; also the rate of uptake observed is the same at both rates of shaking. Beyond this point the velocity becomes limited by diffusion, and practically independent of the amount of yeast, at the slower rate of shaking. At the higher rate of shaking however the respiration rate can be followed up to considerably higher values before diffusion limits the process. It appears therefore that with yeast the apparatus is capable of accurately measuring uptakes up to about 1000 mm.^{3} per hour at the normal rate of shaking (120 per minute), at 15° and with the flasks filled with air. Correspondingly higher velocities can be measured at higher temperatures or with the flasks filled with pure oxygen instead of air.

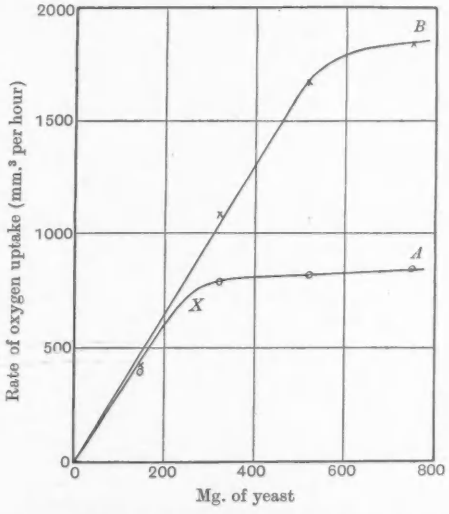

Fig. 5. Velocities of oxygen uptake observed with varying amounts of yeast. A suspension of 5 g. baker's yeast in 20 cc. phosphate buffer p_H 7·6 was prepared, and mixtures of this suspension with phosphate buffer were taken so as to give the amounts of yeast stated in 3 cc. of liquid. In curves A and B the rates of shaking were 102 and 138 oscillations per minute respectively. Temperature 16°.

The maximum rate of uptake which can be measured varies greatly with the rate of shaking, as is to be expected. In order to obtain some idea of this rate of uptake at different rates of shaking, a number of observations were made with larger amounts of yeast, under conditions therefore where diffusion was the limiting factor. These results are shown in Fig. 6. The experimental variations are considerably greater when the process is limited by diffusion than when this is not the case, but the results are quite consistent in spite of the fact that several different respirometers were used. The true respiration rate of the amount of yeast used was about 3000 mm.^{3} per hour

at 15°, as shown by determinations on small amounts with rapid shaking. This rate was reached at a rate of shaking of about 180 oscillations per minute.

Rates of shaking below 60 per minute do not cause much mixing of the liquid layer, which tends to oscillate as a whole from side to side of the flask. Definite mixing of the liquid begins at about 60, and thereafter becomes increasingly efficient. This is reflected in the form of the curve. Rates of shaking above 200 are not permissible, as there is then some risk of the liquid splashing on to the "KOH-paper," but there is no risk of this below this rate.

The position of the curve of Fig. 6 is not altogether invariable and independent of the nature and amount of the system absorbing oxygen. Even when the process is limited by diffusion the rate of uptake depends to a certain extent upon the amount and activity of the yeast taken (or, in the case of

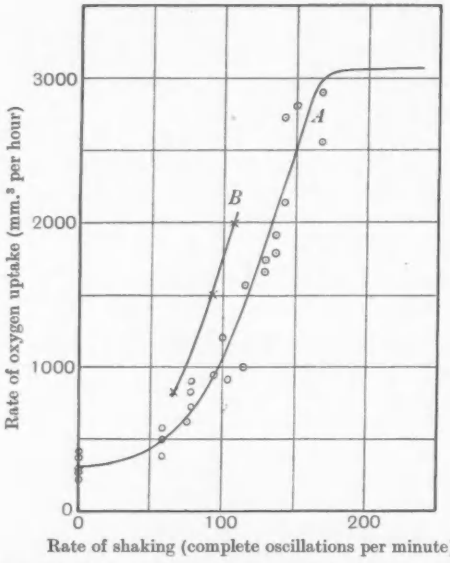

Fig. 6. Effect of rate of shaking on rate of oxygen uptake by large amounts of yeast. Each of the small circles represents an experiment with 600 mg. baker's yeast in 3 cc. phosphate buffer at 15°. The true respiration rate was slightly over 3000 mm.³ per hour. Curve B was obtained with 200 mg. of yeast at 37°.

autoxidation of a substance, the velocity constant of the reaction). In general, systems which would react rapidly with an adequate supply of dissolved oxygen tend to give higher curves than systems giving slower uptakes. That is to say, diffusion effects may influence the results at observed rates much lower in the case of systems giving only small rates of uptake (in the absence of such effects) than in the case of more rapidly reacting systems.

Thus with smaller amounts of yeast than those used in Fig. 6 the curves followed lie somewhat lower but are of similar form. As the rate of shaking

is increased the rate of uptake increases as before until the true respiration rate is reached, after which further increase of shaking has no effect. With decreasing amounts of yeast a series of curves such as E, F, B, C in Fig. 7 are thus obtained, the levels of the horizontal parts of the curves at the higher rates of shaking being proportional to the amount of yeast present. It is of course only on these horizontal parts of the curves that the true respiration rate is measured.

Conversely with more rapidly respiring cells the position of the curve is somewhat higher. In curve D of Fig. 7, for which the same amount of yeast was used as in Fig. 6, the yeast was previously incubated with a small amount of glucose, which increased its respiration about 5 times [cf. Dixon and Elliott, 1929]. The change in position of the curve is however comparatively small.

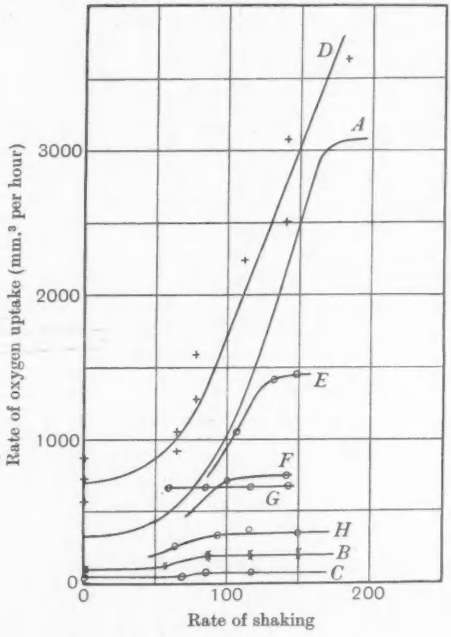

Fig. 7. Curve A is that of Fig. 5 reproduced for comparison. Curves A, B and C were carried out with the same sample of yeast, 600 mg., 60 mg., and 20 mg. being used respectively. Curve D was obtained with 600 mg. of the same yeast which had previously been treated with a small amount of glucose. Curves E and F were obtained with 500 mg. and 250 mg. of a different sample of yeast. Temperature 15° in all these curves. Curve G was obtained with 100 mg. of yeast at 37°, and curve H with 500 mg. of minced liver at 37°.

In the case of yeast the respiration rate is independent of the oxygen pressure within wide limits. In other cases, as for instance the autoxidation of cysteine, the reaction velocity depends upon the oxygen pressure.

A number of experiments were carried out on the oxygen uptake by cysteine in presence of varying amounts of iron as catalyst. The curves obtained were very similar to those given by yeast. It appears that with this system also velocities of the order of 1000 mm.^{3} per hour can be measured at the usual rate of shaking (see Fig. 8).

This figure must not however be taken as representing the highest velocity measurable by the apparatus for all systems. With certain rapid reactions uptake rates of a much higher order can be measured without errors due to

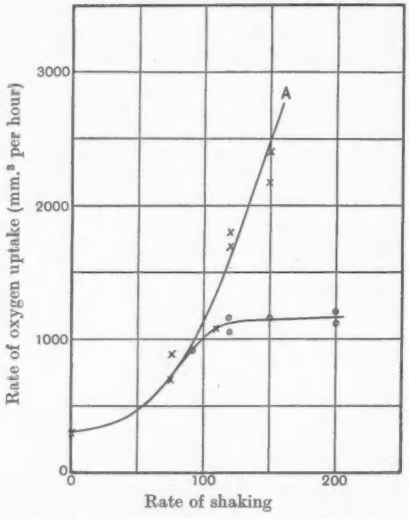

Fig. 8. Effect of rate of shaking on oxygen uptake by cysteine in presence of iron. The curve A is again reproduced from Fig. 6 for comparison. The crosses represent observations with 8 mg. cysteine + 0·04 mg. iron (as $FeSO_4$) in 3 cc. buffer. The circles were obtained with 8 mg. cysteine + 0·016 mg. iron. Temperature 14°.

diffusion effects. For instance in the case of oxygen uptake by alkaline pyrogallol solutions rates of the order of 10,000 mm.^{3} per hour can be measured at the normal rate of shaking.

Fig. 9 shows the rates of uptake observed at different rates of shaking. In these experiments the pyrogallol solution was placed in the flasks, and the NaOH solution was placed in small hanging tubes as shown in Fig. 1. The manometers were then shaken in the thermostat for a few minutes, after which the NaOH was upset into the pyrogallol solution and readings commenced. By this means the rapid uptakes could be conveniently followed.

It will be seen that, although at the slower rates of shaking the reaction was evidently limited by diffusion, the velocity was quite unaffected by increasing the rate of shaking from 120 to 150, indicating that at 120 the true

velocity had been reached. This is also indicated by the fact that the velocity of uptake was exactly proportional to the pyrogallol concentration up to even higher velocities, as shown in Fig. 10. We have at present no wholly satisfactory explanation for the difference in behaviour between pyrogallol and the other systems studied.

It is now clear that the limiting rate of uptake which can be measured in the apparatus without being falsified by diffusion effects does not depend only upon the apparatus, but also upon the system under investigation. Our

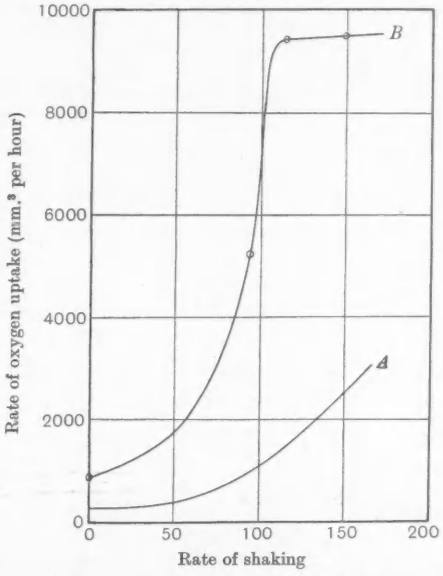

Fig. 9. Effect of rate of shaking on rate of oxygen uptake by alkaline pyrogallol. The curve A is again that of Fig. 6. Curve B was obtained as follows. 2·1 cc. water + 0·5 cc. of 0·5 % pyrogallol solution was placed in the flasks, and 0·4 cc. of 20 % NaOH in the hanging cups. After equilibration of the apparatus the NaOH was upset into the pyrogallol solution. Temperature 14°.

aim in this paper is not to provide a mathematical treatment which will account quantitatively for the effects observed, but rather to call attention to the absolute necessity, when studying any reacting system by means of the apparatus, of making certain that diffusion effects are not affecting the results, if the observations are to have any weight. Two main criteria are available. In the first place, it is obvious that if the reaction velocity is determined for a small amount with rapid shaking this will indicate the true value. If from this the value for a larger amount is then calculated, and the observed value corresponds with the calculated, it is clear that the true rate is still being measured. In other words, if the observed velocity is proportional to the amount of the system taken throughout the experimental range,

the experiments are valid. The second requirement is that the velocity of uptake must not be affected by the rate of shaking. Unless this is the case it is clear that diffusion effects are entering, and the significance of the results is doubtful. In studying a new system it cannot be predicted from the observed velocity alone whether diffusion effects are influencing the results or not, and one or other of the tests mentioned must be applied.

It can however be stated with some probability that with this type of apparatus at the usual rate of shaking and for reactions of average velocity,

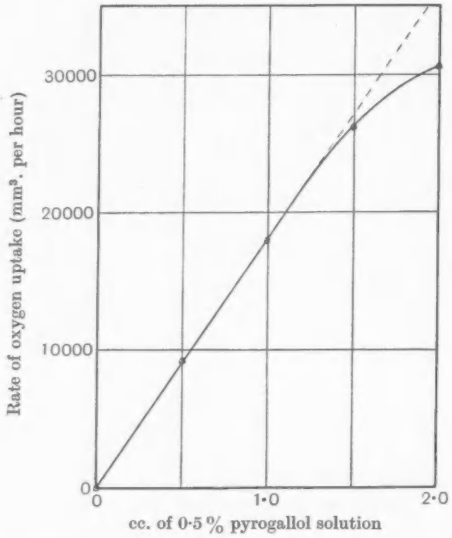

Fig. 10. Effect of amount of pyrogallol used on rate of oxygen uptake. The procedure was the same as in Fig. 9, except that the rate of shaking was maintained constant at 120, and the volume of 0·5 % pyrogallol solution taken was varied. The total volume of liquid was 3 cc. in each case as before.

diffusion effects do not play a considerable part with rates of uptake less than about 1000 mm.³ per hour at 15°. An examination of the literature shows that the rates of uptake measured with this type of apparatus have almost invariably been considerably lower than this—usually about 200–300 mm.³ per hour—and in that case it is unlikely that any considerable errors due to this cause have arisen. It is however not altogether uncommon in descriptions of work on oxygen absorption carried out in other types of apparatus to find such statements as "In order to obtain consistent results it was necessary to control carefully the rate of shaking." This of course indicates that such experiments are of doubtful significance.

Finally we may mention that none of our earlier work is affected by these results. We have always applied one or other of the above-mentioned tests

to ensure that diffusion errors were eliminated, and when we have worked with systems producing CO_2 we have employed the "KOH-paper" technique. Nevertheless in view of the large amount of work now being carried out with the apparatus it seemed desirable to publish the facts described in this paper.

SUMMARY.

1. The usual method for absorbing the CO_2 evolved in respiration experiments in the Barcroft apparatus is shown to be very inefficient, and an efficient method is described.

2. Experiments on the extent to which diffusion effects may limit the results obtained, when the Barcroft apparatus is used for measuring velocities of oxygen uptake, are described and discussed.

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XCII. THE OXYGEN CAPACITY OF THE BLOOD IN ONE HUNDRED CASES OF MENTAL DISORDER.

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It is probable that the most important factor responsible for the abnormal reactions in cases of mental disease can be traced to some variety of defective oxygen metabolism [Pickworth, 1929].

Irregularities of the normal oxygen supply or its utilisation may occur at any point in the respiratory cycle. The present paper deals with deficient oxygen supply due to defective blood-pigment. From pathological considerations it is highly probable that toxic substances absorbed from the intestinal canal (and evidence of blood infection with intestinal organisms is found almost invariably in association with acute mental disorder) might effect a chemical change in the haemoglobin of the blood, rendering it useless for respiratory purposes.

The present investigation was therefore undertaken to ascertain whether inactive forms of haemoglobin occurred in the blood in cases of mental disorder.

The active haemoglobin of a large number of specimens was determined by direct measurement of the oxygen liberated from fully oxygenated blood, and these values were compared with the total haemoglobin content found by other methods of analysis.

Since it was required to measure very small quantities of inactive pigment, it was necessary to devise an accurate and reliable method of analysis specially suited to the work and quantity of material available. A modification of Haldane's blood-gas apparatus [1900, 1920] was made which was adaptable for a small quantity of blood. Control experiments of each factor influencing the analysis were made in order to secure consistency and reliability of the results.

Methods.

Estimation of the oxygen capacity of blood.

The blood was collected in sterile tubes containing a trace of potassium oxalate and sodium fluoride as an anti-coagulant. Carbon dioxide was removed by exposing in films to a current of moist, carbon dioxide-free air in presence of a phosphate buffer solution of p_H 7·5. A measured quantity

(1 to 2 cc.) of this CO_2-free blood-buffer mixture was introduced into the reaction flask of the apparatus; this was immersed in a water-bath at 37° and gently shaken until the blood had attained equilibrium with the atmosphere in the flask. 1 cc. of ferricyanide with saponin was then admitted from the separate compartment of the apparatus into the reaction flask. The oxygen was completely expelled by shaking the apparatus for 3 minutes, and the volume of gas evolved measured and calculated to 100 cc. of blood.

Description of blood-gas apparatus.

The apparatus consisted of a narrow bore U tube containing clove oil tinted with safranine as a pressure indicator; there was a third limb of wider bore acting as a reservoir for the oil which was also convenient for measuring the gas under definite barometric pressure.

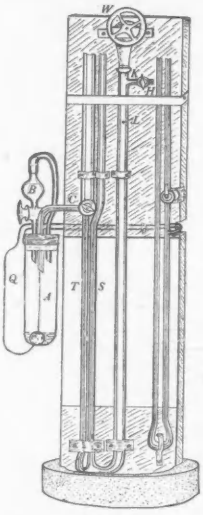

Fig. 1.

The burette *T* (Fig. 1) was fitted with a two-way tap by means of which it could be connected either with the side limb *C* and the reaction flask *A*, or opened to air for adjustment to barometric pressure.

The ferricyanide was contained in a separate compartment *B*, which was connected above with the reaction flask so that it could be admitted without altering the pressure. The barometric pressure and temperature control bulb *Q* was of similar size (about 30 cc.).

The burette tubes *T* and *S* were of identical bore, the former being accurately graduated to a volume of exactly 0·250 cc. and divided into 100 equal parts.

The apparatus was rigidly mounted on a very heavy base and was designed for use completely immersed in a water-bath at 37°. The temperature was controlled by a thermostat and the water was kept thoroughly stirred by a current of air.

The rigid nature of the connections in the apparatus was an important feature and allowed thorough shaking in order to mix the reacting fluids. Complete and rapid expulsion of the liberated oxygen was secured by the introduction of a single large glass marble 1·5 cm. in diameter into the reaction flask.

Accurate and reliable analyses could be made with as little as 0·5 cc. of blood.

A. *Collection and preservation of specimens.*

The blood was collected in sterile tubes containing 0·02 g. of a finely powdered mixture of potassium oxalate and sodium fluoride, 5 : 1.

This anti-coagulant did not cause haemolysis. Estimations were carried out immediately after collection of the specimens when possible. Otherwise

they could be stored satisfactorily for a short period in an ice-chamber. It was found that sterile blood, so kept, did not change in oxygen capacity in 4 days, although after 6 days an appreciable diminution was noticeable.

B. *Preliminary treatment of blood samples.*

In order to eliminate errors caused by varying hydrogen ion concentrations of the blood specimens, and also to prevent liberation of carbon dioxide during the test, the p_H of the blood was controlled by means of the addition of strong buffer solution, and the carbon dioxide was removed from the mixture before introducing it into the flask. An equal volume of phosphate solution of p_H 7·5 (containing 8·184 g. $Na_2HPO_4, 12H_2O$ and 0·608 g. NaH_2PO_4 in 50 cc. of distilled water) was added to the blood. A steady stream of air purified from traces of CO_2 by bubbling through dilute sodium hydroxide was passed over the mixture which was gently rotated in a tonometer. This treatment removed about 20 cc. of CO_2 per 100 cc. of blood in 20 minutes. Analyses conducted with the blood so treated were proved to be quite consistent.

C. *Equilibration of samples at 37°.*

For each estimation, 1·6 cc. of the blood-buffer mixture was introduced into the flask *A* by means of a standardised delivery pipette; approximately 1 cc. of recently made ferricyanide (12·5 % dissolved in the same buffer solution, diluted five times in order to bring the salt concentration to a suitable amount), with 0·005 g. of white saponin, was placed in the bulb *B*.

In order for the blood to attain complete equilibrium with the air of the flask, it was necessary to shake the apparatus in the bath for 15 minutes, equilibrium being evidenced by the clove-oil levels remaining quite stationary for 2 minutes.

D. *Process of analysis.*

The levels of the clove oil in the burette tubes were brought to a convenient position near to the zero point by adjusting the pressure in the tube *L*, the temperature gauge was closed, and the ferricyanide solution admitted. The apparatus was gently shaken for 3 minutes. The large glass marble greatly facilitated rapid expulsion of the liberated oxygen in a manner much superior to a number of smaller beads, or broken glass. The levels were adjusted to the gas increase and the temperature of the bath was noted. After obtaining equilibrium the volume of gas was observed and calculated to N.T.P. per 100 cc. of blood. A further shaking of the apparatus was then carried out which should cause no further evolution of gas.

Accuracy of the analysis. We were able to eliminate the following errors by a careful investigation into the conditions under which the test was carried out, and in every case considerable latitude could be allowed in the quantities and times of the tests without introducing these or other errors.

Time in minutes

(*a*) Incomplete liberation of oxygen due to faulty haemolysis of the corpuscles (see experimental section E′).

(*b*) Blood laked with dilute ammonia may retain a portion of the liberated oxygen.

(*c*) CO_2 may be liberated owing to the insufficient alkalinity of the solution.

(*d*) Litarczek [1928], and Parsons and Parsons [1927] have described a reducing action which occurs in specimens of pathological blood which results in an absorption of some of the oxygen evolved in the test.

Our experiments under similar conditions demonstrated that some specimens of human blood absorbed a quite appreciable amount of the liberated oxygen. It was found that plasma or serum from normal animals reacted similarly, and even washed rabbit or sheep corpuscles possessed this reducing property to a slight degree. These errors were eliminated by adequately buffering the test mixture, the advantage of which is illustrated by experiments in section B′ and Figs. 2 and 3.

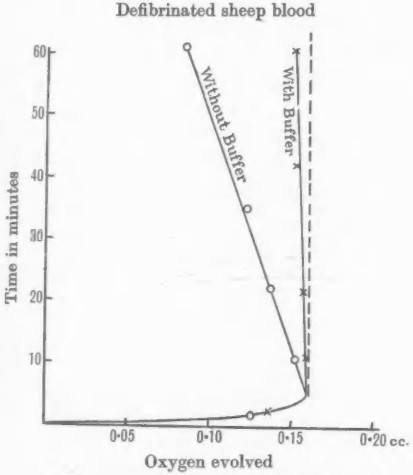

Fig. 2. To show effect of buffer in reducing oxygen absorption in test.

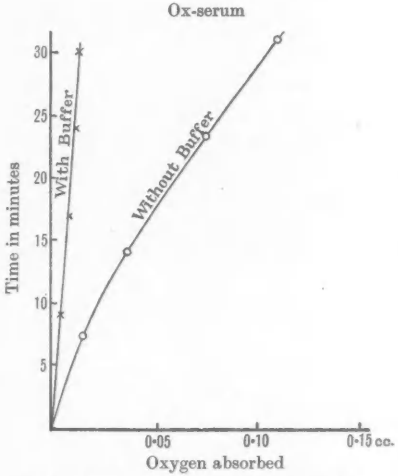

Fig. 3. To show effect of buffer in reducing oxygen absorption in test.

The accuracy of the method was carefully checked by other tests which included Stadie's cyanohaemoglobin colorimetric method, and an estimation of the iron content of the haemoglobin by a micro-titration method using titanium chloride. The quantity of haemoglobin was also estimated by the Fleischl-Miescher haemoglobinometer using a calibrated carbon monoxide wedge.

Experimental.

A′. *Preservation of specimens.*

The following typical experiment demonstrates the change in the oxygen capacity of blood containing oxalate-fluoride anti-coagulant, after storing in the ice-box for various periods.

Specimen No. 59. Sterile rabbit blood + 0·5 % oxalate-fluoride mixture.

Time of storing	Oxygen capacity per 100 cc.
Fresh	15·1 cc.
24 hrs.	15·0 „
48 hrs.	15·0 „
4 days	15·0 „
6 days	12·3 „

No change occurred in 4 days, after which a distinct fall in oxygen capacity took place.

B′. *Effect of buffering the sample.*

1. A large number of sera from various sources, when mixed with an equal quantity of phosphate buffer, were found to have a p_H of 7·5 by the capillator method. The clear fluid obtained by centrifuging a mixture of equal parts of blood and buffer also had a similar p_H value.

2. No alteration in the volume of gas, due to changes in vapour pressure, was caused by the addition of buffered ferricyanide to blood-plasma plus an equal volume of buffer.

Exp. 80. 0·81 cc. human plasma + 0·81 cc. buffer.

Time	Burette reading
Equilibrium	0·185 cc.
3 mins.	0·185 „
Ferricyanide added	
7 „	0·185 „
15 „	0·185 „

3. The addition of acid or alkali to blood did not affect the oxygen capacity, provided the buffer solution as described above was added before saturation with air previous to the test.

Exp. 82. 1 cc. blood + 0·5 cc. 2 % Na_2CO_3; oxygen evolved after buffering etc. = 0·120 cc.

1 cc. blood + 0·5 cc. 0·3 % lactic acid; oxygen evolved after buffering etc. = 0·120 cc.

1 cc. blood + 0·5 cc. isotonic saline; oxygen evolved = 0·121 cc.

4. The addition of a small amount of lactic acid to blood has a very marked effect at low oxygen pressures; when this lactic acid-blood is treated with buffer prior to saturation with the low pressure of oxygen, the calculated theoretical result is obtained.

Exp. 83. 1 cc. corpuscles + 1 cc. isotonic saline + 0·5 cc. 0·1 % lactic acid exposed to 2·6 % oxygen in nitrogen.
Oxygen evolved = 0·194 cc. = 67·5 % saturated.
(Calculated saturation = 84 %.)
1 cc. corpuscles + 0·5 cc. lactic acid + 1 cc. buffer solution, then exposed to 2·6 % oxygen in nitrogen.
Oxygen evolved = 0·225 cc. = 84 % saturated.
(Calculated saturation = 84 %.)

5. The effect of the buffer in preventing the re-absorption of oxygen by the blood-ferricyanide mixture is shown by the following experiment.

Exp. 85. 1 cc. sheep plasma + 1 cc. 2 % Na_2CO_3, after equilibrium established with its atmosphere in the reaction flask, 1 cc. aqueous ferricyanide added. Readings taken at intervals.

Time	Burette reading	Time	Burette reading
0 mins.	0·229 cc.	12 mins.	0·193 cc.
2 „	0·220 „	18 „	0·183 „
5 „	0·212 „	25 „	0·170 „
8 „	0·200 „	30 „	0·162 „

Absorption in 30 mins. = 0·067 cc.

Exp. 86. 1 cc. sheep plasma + 1 cc. buffer p_H 7·5; after equilibrium established, 1 cc. buffered ferricyanide added; readings at intervals.

Time	Burette reading	Time	Burette reading
0 mins.	0·190 cc.	15 mins.	0·188 cc.
5 „	0·189 „	30 „	0·188 „
10 „	0·189 „	40 „	0·187 „

Oxygen absorbed = 0·003 cc.

Similar tests comparing alkaline with buffered solutions are shown in Figs. 2 and 3.

It will be noted that the serum is responsible for the greatest re-absorption of oxygen. A further similar test with washed sheep corpuscles gave a much smaller absorption deviation.

C'. *CO_2 in the sample.*

When considerable amounts of carbon dioxide are present in the blood sample, a portion of it passes into the air above the mixture in the reaction flask. This quantity may vary slightly on adding the reagent, and samples must therefore be previously treated to remove excess CO_2.

Exp. 87. (*a*) 1 cc. sheep blood-plasma previously exposed to CO_2-free air current for 20 minutes (CO_2 content = 20 cc./100 cc.); 1 cc. buffer added; ferricyanide admitted when in equilibrium.

	Time	Burette reading
	0 mins.	0·147 cc.
	3 „	0·147 „
	Equilibrium established	
Ferricyanide added	5 mins.	0·147 cc.
	10 „	0·146 „
	25 „	0·146 „

No change on adding reagent.

(*b*) 1 cc. sheep plasma (untreated), CO_2 content = 40 cc./100 cc., 1 cc. buffer added; ferricyanide admitted when in equilibrium.

Time	Burette reading
0 mins.	0·135 cc.
3 „	0·135 „
Ferricyanide added	
5 „	0·135 „
8 „	0·120 „
10 „	0·116 „
12 „	0·116 „

When blood-plasma containing a considerable quantity of carbon dioxide is used, there is a distinct change in the volume of the gas on addition of the reagent, due to absorption of CO_2.

Exp. 88. The quantity of CO_2 in the atmosphere of the reaction flask is shown in the table below. Blood + equal volume of buffer p_H 7·5 exposed for varying periods to a current of moist, CO_2-free air; 2 cc. of the resulting mixture introduced into the apparatus. After the volume had become constant, the CO_2 in the flask was absorbed by admitting 0·2 cc. of 5 % sodium hydroxide, the contraction in volume being the amount of CO_2 present.

Time of exposure to CO_2-free air	CO_2 in gas of reaction flask	Equivalent mm. of CO_2
0 mins.	0·57 %	3·9 mm.
5 „	0·31 %	2·2 „
10 „	0·21 %	1·5 „
20 „	0·14 %	1·0 „
30 „	0·13 %	0·91 „
60 „	0·12 %	0·85 „

Laboratory air = 0·4 mm. CO_2.

Exp. 89. Elimination of excess CO_2. The blood at p_H 7·5 was exposed in a tonometer through which a current of CO_2-free air was passed for varying periods.

Time of exposure	CO_2 per 100 cc. blood
0 mins.	42·5 cc.
1 „	37·5 „
5 „	28·0 „
15 „	22·5 „
20 „	21·2 „
30 „	20·0 „
3 hrs.	17·5 „

An exposure of 20 minutes was sufficient to remove the excess CO_2.

D′. *Equilibration of gas pressures.*

It was found that whole blood required about a quarter of an hour to attain equilibrium with the air of the reaction flask, gentle shaking of the apparatus being continued during the whole time. This period was much increased for plasma, whilst only a few minutes were required for a suspension of washed blood-corpuscles in saline.

E′. *The ferricyanide-saponin reagent.*

This consisted of 12·5 g. ferricyanide + 0·2 g. white saponin in 100 cc. phosphate buffer diluted five times with distilled water.

1. *Amount of ferricyanide.* A large excess of the ferricyanide mixture is necessary in order to decompose the oxyhaemoglobin completely. (Conant and Fieser [1924] have calculated that at least 13·5 equivalents per g.-molecule of haemoglobin are required when in neutral solution.)

The following test shows that the amount used (0·125 g. for each estimation) was amply sufficient, and that this amount could be varied considerably either way without error.

Exp. 90. (1) 2 cc. buffered ox blood + 1·5 cc. ferricyanide reagent: oxygen evolved = 0·216 cc.
(2) 2 cc. buffered ox blood + 1·0 cc. ferricyanide reagent: oxygen evolved = 0·216 cc.
(3) 2 cc. buffered ox blood + 0·5 cc. ferricyanide reagent: oxygen evolved = 0·216 cc.

2. *Amount of saponin.* The addition of an amount of saponin equivalent to that in the reagent (*i.e.* 0·002 g. per cc.) to a sample of blood caused complete haemolysis in 2 minutes. Microscopic examination of a drop of the fluid failed to show the presence of corpuscles. The addition of strong salt solution to this clear fluid did not cause the re-appearance of corpuscles (microscopically or by turbidity) as occurs with blood laked with a small quantity of distilled water.

The following test shows that the accuracy of the analysis was not affected if the amount of saponin added to the blood varied considerably from the quantity actually employed.

Exp. 92. (1) 2 cc. buffered ox blood + 1·0 cc. ferricyanide containing 0·4 % saponin: oxygen evolved = 0·203 cc.
(2) 2 cc. buffered ox blood + 1·0 cc. ferricyanide containing 0·2 % saponin: oxygen evolved = 0·203 cc.
(3) 2 cc. buffered ox blood + 1·0 cc. ferricyanide containing 0·15 % saponin: oxygen evolved = 0·203 cc.

The rate of absorption of oxygen by haemoglobin was markedly slower in the presence of saponin.

Exp. 93. (1) Human blood exposed to 2·1 % oxygen. Then exposed to air for 15 mins.
Oxygen content = 17·8 cc. per 100 cc. = 100 % saturated.
(2) Human blood exposed to 2·1 % oxygen; saponin added. Exposed to air for 15 mins. exactly as in test (1).
Oxygen content = 16·4 cc. per 100 cc. = 92 % saturated.

Other methods of estimation of haemoglobin content.

1. *Cyanohaemoglobin colorimetric determination.* A slight modification of the process described by Stadie [1920] and Van Slyke and Stadie [1921] has been utilised. The pigments are converted to methaemoglobin by adding a trace of ferricyanide to the diluted blood and then, by adding a little dilute

potassium cyanide solution, cyanohaemoglobin is produced which has a brownish-red colour suitable for colorimetric estimation. A control is used, similarly prepared from pure blood. Blood containing small amounts of methaemoglobin shows a higher value for the colorimetric, as compared with the gasometric, method.

0·2 cc. blood is diluted to 5 cc. with distilled water; 1 drop of 10 % ferricyanide containing 2 % lactic acid is added. After standing for 15 mins., 1 cc. 0·1 % KCN is added and the solution diluted to 20 cc. The resulting colour is matched with the standard similarly prepared.

The lactic acid greatly facilitates the rate of conversion to methaemoglobin, and the colour is stable and unaffected by the amount of acid present.

2. *Fleischl-Miescher haemoglobinometer method.* A thin film of blood was exposed to coal gas in a tonometer until saturated with carbon monoxide. It was diluted 1 : 200 in a haemocytometer pipette with water saturated with carbon monoxide and the solution placed in one-half of the comparing chamber, the other half being filled with distilled water. The haemoglobin values were read directly from a carbon monoxide wedge.

3. *Micro-titration of the iron content.* This method of analysis was used to check the other determinations and is similar to that used by Peters [1912].

The blood was weighed into a small nickel crucible (about 0·3 g.) and ashed by direct heat. The residue was dissolved in 2 drops of distilled water and 1 drop of strong iron-free hydrochloric acid. A drop of dilute hydrogen peroxide was added, the solution diluted to about 0·5 cc. and evaporated almost to dryness in order to ensure decomposition of all traces of peroxide. The solution was transferred to a titration tube having a constricted neck and bulb of capacity 3 cc. with a little acidulated water, and the volume of solution and washings made up to 2 cc. The titrations were made from a micro-burette, capacity 1 cc., with approx. $N/200$ titanium chloride. The reagent was made by adding 15 cc. of 15 % commercial titanium chloride to 15 cc. of hydrochloric acid, boiling for 3 mins. and diluting to 2500 cc. with well-boiled water. This volume completely filled the containing vessel.

It was stored in contact with an atmosphere of CO_2, and standardised immediately before making analyses.

In order to prevent re-oxidation by the oxygen in the titration tube, the air was completely displaced before the analysis by means of a rapid stream of CO_2 and the surface of the liquid was covered with a thin layer of liquid paraffin.

One drop of 10 % KCNS was used as indicator and the titanium chloride was added from the micro-burette until the red colour just disappeared. When the ashing process was correctly performed and the precautions indicated above were observed, no return of the red tint occurred for some time and accurate analyses were obtained.

Results.

Blood specimens from a number of normal healthy persons and 130 specimens from 100 patients have been analysed. Also 40 specimens from healthy animals, including the sheep, ox, pig and rabbit, have been determined, and a number of analyses have been made with the blood of cats and rabbits whilst under treatment with drugs.

All the analyses were performed in duplicate, the results agreeing to within 2 % for the colorimetric tests and 1 % with the other determinations. The following is a summary of the results obtained. Values have been expressed on the basis of 18·5 cc. oxygen = 100 % haemoglobin (Haldane's standard for normal male).

A. *Normal human blood.*

The following tables show the results obtained in analyses of the blood of a number of healthy persons. A repeat of No. 1 is included to show the consistency of results obtainable after a few hours' interval.

Table I. *Oxygen capacity (normal human specimens).*

Specimen	Oxygen evolved per 100 cc.	Corresponding % Hb. content
1	19·0 cc.	102·6
2	18·6 „	100·7
3	17·6 „	95
4	18·4 „	99·5
1 (repeat)	19·05 cc.	102·9

Table I *a*. *Haemoglobin content (comparison of methods).*

Specimen	% Hb. by gasometric method	% Hb. by Stadie method	% Hb. by CO-Hb. method
1	102·6	103	102
2	100·7	101	100
3	95·1	Standard	94
4	99·5	99·2	98

Table II gives the results of analyses of a number of specimens from healthy animals.

Table II. *Haemoglobin content of animals' blood.*

Animal	% Hb. by gasometric method	% Hb. by $TiCl_3$ titration	% Hb. by CO-Hb. method	% Hb. by Stadie method
Sheep	108	106·5	106	108
Ox	86·5	86·8	87	Standard
Pig	71·4	72·2	70	71·2
Ox (corpuscles)	99·4	—	99	99·3
Rabbit	59·5	—	59	59·2
Cat	57·8	—	58	57·2
Sheep	110·8	109	110	110·7
Pig	84·3	86	85	84

The values calculated from the gasometric method agreed very closely in all cases with those by the cyanohaemoglobin colorimetric method. The values by the Fleischl-Miescher method varied slightly owing to a greater difficulty

in matching the colour. (The tints of the blood solution, when fully saturated with carbon monoxide, compared satisfactorily with the standard wedge with the exception of rabbits' blood which gave a yellower tint.) Determinations of the exact colours were also made by the Rosenheim-Schuster colorimeter, and some of the results are given which may possibly be useful for reference.

Colour values of haemoglobin solutions.

Normal human blood diluted 1 : 100

Specimen	Tintometer units
Oxyhaemoglobin	Red 15·0; yellow 1·5
Carboxyhaemoglobin	Red 20·2; yellow 0·2
Cyanohaemoglobin	Red 12·0; yellow 4·2

B. *Patients' blood.*

The absolute haemoglobin content as found by the gasometric method is given in Table III.

Table III. *Haemoglobin content of patients' specimens.*

	Males		Females	
Hb. content	No. of specimens	%	No. of specimens	%
Over 100 %	6	16	9	10
90–100 %	20	53	30	32
80– 90 %	9	23	39	42
70– 80 %	3	8	11	12
Below 70 %	0	0	3	3

In only comparatively few cases was there any serious deficiency in the quantity of available haemoglobin. The average haemoglobin content for the whole series was 93 % for males and 89 % for females.

Table IV gives the results in 20 unselected cases, 10 male and 10 female.

Table IV.

Sex	Oxygen from 100 cc. blood cc.	% Hb. by oxygen evolved	% Hb. by cyano-Hb. method	% Hb. by CO-Hb. method	Notes
M.	16·2	87·6	88	86	—
,,	16·1	87·0	88	88	—
,,	17·6	95·3	95·6	95	—
,,	16·5	89·2	96·5	96	7·3 % inactive Hb.
,,	18·0	97	97	98	—
,,	19·8	107	107	108	—
,,	17·5	95	94	93	—
,,	17·0	92	98	96	6 % inactive Hb.
,,	17·1	92·6	93	93	—
,,	16·6	90·0	91	90	—
F.	17·0	92	92	92	—
,,	16·0	86	86	86	—
,,	15·9	86	87	87	—
,,	20·1	108·5	110	110	1·5 % inactive Hb.
,,	15·5	84	84·8	85	—
,,	19·6	106	106	106	—
,,	16·4	88·5	92	91	3·5 % inactive Hb.
,,	17·5	95	93·5	95	—
,,	15·9	86	86	86	—
,,	16·5	89	89	88	—

Three of the above specimens gave low results by the oxygen method as compared with the colorimetric and titrimetric methods; this indicates the presence of inactive haemoglobin.

Among the total 100 cases, 28 showed deficient oxygen capacity compared with the theoretical. In all these the difference exceeded 2·5 %, the greatest deficiency being 12·5 %, which was observed in two specimens; neither of these patients had received treatment with sulphonal or allied drugs.

In accordance with previous supposition of the effect of intestinal disease, the 100 cases contained a large number whose blood showed a positive agglutination by the Widal test. (See Table VI.)

A number of cases showing deficient active haemoglobin were examined at intervals of several weeks. In most cases the deficiency persisted for a long period. The following table is included to illustrate this important finding.

Table V. *Haemoglobin in blood of special cases.*

Name	Date	Sex	% Hb. by gasometric method	% Hb. by colorimetric method	% difference
H.F.	14. iii. 29	F.	95	100	5
„	3. v. 29	„	89	97	8
„	30. v. 29	„	99·3	109	9·7
„	14. vi. 29	„	101	113	12
A.S.	24. v. 29	„	72·6	83	10·4
„	14. vi. 29	„	74	80	6
A.J.	14. vi. 29	„	80	86·5	6·5
„	16. v. 29	„	71·5	82	10·5
B.W.	19. vii. 29	„	90	99	9
„	25. vii. 29	„	81	87·3	6·3
„	30. viii. 29	„	85·1	92	6·9
„	5. ix. 29	„	91	102·8	11·8
D.G.	30. viii. 29	„	82·4	92	9·6
„	5. ix. 29	„	84	89	5
„	18. ix. 29	„	101·3	107·1	5·8
L.S.	1. viii. 29	„	90	100·8	10·8
„	5. ix. 29	„	79	88	9
D.R.	5. vii. 29	M.	92	98	6
„	2. viii. 29	„	92·5	98	5·5
R.P.	27. vi. 29	„	89·2	96·5	7·3
„	2. viii. 29	„	85	91	6·0
J.G.	5. ix. 29	„	95	107	12·0
H.H.	26. ix. 29	„	103	110	7·0

Table VI.

Widal agglutination reaction	No. of cases	Cases with over 2 % of inactive pigment	Average % of inactive pigment
+	52	27	4·2
−	38	1	0·38

A simple test for methaemoglobin.

The following test demonstrates the presence of methaemoglobin when 5 % or more is present in blood; with the simple type of spectroscope about 20 % of methaemoglobin must be present for reliable detection.

0·20 cc. of blood is saturated with carbon monoxide (coal-gas) in a tonometer and then diluted with 40 cc. water saturated with carbon monoxide. 10 cc. are transferred to each of two similar tubes and 1 drop of 2 % ammonia

is added to each. The air is displaced by coal-gas from the tubes, which are corked and shielded from bright light. To one tube 0·5 cc. of 1 % KCN is added, and to the other 0·5 cc. of freshly made solution of 1 % sodium hydrosulphite, avoiding undue exposure to air. The stoppers are replaced and the tubes gently inverted. The methaemoglobin of the one tube is converted to brownish-red cyanohaemoglobin, and the methaemoglobin in the other is first reduced to haemoglobin followed by formation of carbon monoxide-haemoglobin. The colour difference is recognised by naked-eye comparison. The colours produced were quite stable when air was excluded.

Discussion.

The presence of inactive haemoglobin has been found to occur in one quarter of the cases examined, the amount in two cases being as much as 12 %. It is submitted that the amount of inactive haemoglobin under certain circumstances is very much higher than this, and further work is being continued with acute cases.

The presence of inactive haemoglobin has been correlated with diseased conditions of the intestinal tract. Such diseased conditions are very frequent in cases of mental disorder, and evidence of blood infection apart from clinical disease can be obtained from the agglutination reaction of the blood in about one quarter of the cases admitted to the mental hospitals investigated (Birmingham) [Pickworth, 1927, 1928].

Table VI shows the relation of inactive haemoglobin to a positive agglutination reaction. The close correlation is obviously more than coincidence and tends to confirm the view that an infected intestinal tract allows the absorption over a large area of bacteria and toxic substances which deprive the blood of its primary function, that of oxygenation. Other visceral conditions of focal sepsis of the upper respiratory tract, the fatty liver and kidneys and the atheroma of the blood vessels, so often found in cases of mental disorder, support this view.

Buscaino [1923, 1926] has also described lesions of the small intestine and holds that a toxic substance is absorbed, especially in acute confusional insanity and in dementia praecox. He suggests that in these cases the liver does not function normally and that amino-acids become decarboxylated, giving rise to poisonous amino-compounds. Stewart [1929] suggested that this toxic compound is tyramine and demonstrated that tyrosine was destroyed with production of the amine by the intestinal bacteria from cases of acute insanity.

Boyd [1923] found histamine in the intestines of children dying from intestinal toxaemia. Looney [1924] has described toxic amines in the blood of mental patients in the depressed phase.

A number of animal experiments have been conducted with a view to reproducing some of these conditions.

1. *Effect of chemical reagents.* (*a*) A rabbit, 2000 g., was injected intravenously with the following quantities of sodium nitrite at intervals of 30 minutes: 0·25, 0·5, 1·0, 1·5, 2·0 cc. of 2 % solution, 2·0 cc. of 5 % solution. Blood samples were taken after 12 hours. The haemoglobin value was 70 %, but no inactive haemoglobin was demonstrated. (Sodium nitrite readily produces methaemoglobin *in vitro*.) This experiment shows how quickly methaemoglobin disappears from the blood. Haldane found that methaemoglobin disappeared in about 4 hours.

(*b*) A rabbit, 1500 g., was injected intravenously with 5 cc. of 5 % sodium nitrite solution. A blood sample taken 40 minutes later contained 64·2 % of blood-pigment by the Stadie method. Only 21·6 % was oxyhaemoglobin, the capacity being 4·0 cc. O_2 per 100 cc. Thus 42·6 % of methaemoglobin was present. The blood was dark and when saturated with carbon monoxide yielded a brown-coloured solution. The animal died shortly after.

(*c*) A cat, 900 g., was given 5 grains phenacetin by mouth on five successive days. The total haemoglobin content of samples taken after the last dose was 45 %, and the active pigment amounted to only 34 % (8·4 cc. oxygen per 100 cc. blood). Thus 11 % of inactive material was present. The blood was dark and the animal anaemic. The presence of the inactive pigment was also demonstrated by the special test described. (Young [1926] asserts that aniline and certain derivatives do not produce methaemoglobin when administered to animals and that the dark colour of the blood in such cases is due to *p*-aminophenol.)

2. *Effect of bacterial injections.* (*a*) Experiments were made in which emulsions of *Streptococcus mitis* and *S. faecalis* were injected intravenously into rabbits. In neither case was there any indication of inactive pigment in the blood.

(*b*) An emulsion of 0·2 cc. of *B. nitrosobacter* was injected intravenously into a rabbit. A specimen of blood taken 2 days later had an oxygen capacity of 11·7 cc. O_2 per 100 cc., *i.e.* 63 % of the normal Hb. content. The total haemoglobin by the Stadie method was 70 %, *i.e.* 7 % of inactive haemoglobin was present.

(*c*) The following injections of T.A.B. vaccine (2000×10^6 organisms per cc.) were made intravenously into a rabbit of 2000 g. weight. 3 cc. on 3. xii. 29, 6 cc. on 9. xii. 29, 12 cc. on 17. ix. 29, 20 cc. on 23. ix. 29. After each injection samples of blood were examined, but in no specimen was inactive pigment demonstrated. In spite of the enormous doses the animal remained apparently well.

(*d*) A rabbit, 2000 g., was injected with 5 cc. of emulsion of *B. enteritidis* Gaertner (20×10^9 organisms per cc.). A blood sample taken 24 hours after the injection contained 15 % of inactive haemoglobin, the active oxyhaemoglobin being 54 %. The animal died 40 hours after the injection.

The nature of the inactive pigment.

The chemical properties of the inert pigment are similar to those of methaemoglobin, and differences, if they exist, can only be ascertained by complicated quantitative spectroscopic analysis. It does not yield oxygen to ferricyanide or a vacuum, is unaffected by carbon monoxide, and has a brownish colour; it is reduced by sodium hydrosulphite.

The occurrence of similar pigment in pathological blood has been described by a number of workers. Harrop [1919], in describing two cases of pneumonia, concluded that methaemoglobin was responsible for the deficiency in oxygen capacity. Haldane [1929] attributed the fatal character of the influenza epidemic of 1918 to the production of methaemoglobin by the infecting organisms.

Parahaematin or cathaemoglobin has been described by Arnold [1900], v. Klaverin [1901] and by Keilin [1926]. This substance does not yield oxygen to a vacuum or to ferricyanide, but possesses a spectrum very similar to oxyhaemoglobin.

Loeb, Bock and Fitz [1921] describe two cases of nitrobenzene poisoning in which the oxygen capacity by the colour should have been 20 cc. O_2 per 100 cc. blood. Actually the values 6·2 and 8·9 cc. were obtained, but no methaemoglobin could be detected spectroscopically. Peabody [1913] also found a fall in the oxygen capacity of guinea-pigs after injection with massive doses of pneumococcus. No spectroscopic evidence of methaemoglobin could be found.

Ray [1928], Ray and Stimson [1927], Stimson [1927], and Stimson and Houbitz [1928] have observed an inactive substance in the blood of rabbits and dogs after removal of the spleen or 80 % of the liver and also following administration of nitrobenzene into the stomach. The maximum amount found was 15 %. It disappeared from the blood after 7 hours in the nitrobenzene experiments and not until 5 days following splenectomy.

Carbon monoxide-haemoglobin, sulph-haemoglobin, and nitric oxide-haemoglobin, which are useless for respiratory purposes, have been sought for but were not found in any of the specimens.

Summary.

The oxygen capacity of blood has been compared with the total haemoglobin content in order to find out whether derivatives of haemoglobin, useless for respiratory purposes, occur in the blood of mental patients.

A special form of apparatus of the Haldane type has been devised, yielding results of greater accuracy and reliability than heretofore.

The haemoglobin was determined by the Stadie cyanohaemoglobin method, the Fleischl-Miescher haemoglobinometer and a titration of the actual iron content by the titanium chloride method.

130 specimens from 100 cases have been examined, and in 28 cases deficient oxygen capacity has been recognised. The difference between the active and total haemoglobin varied from 2·5 to 12·5 %.

The presence of inactive pigment can, in many instances, be correlated with positive Widal agglutination reactions to organisms of the enteric group, and these infections may prove of importance in the aetiology of this pigment.

The above work has been carried out in the laboratories of the Joint Board of Research for Mental Diseases, City and University of Birmingham.

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